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THE ECOLOGY AND EVOLUTION OF RARE, SOIL SPECIALIST *ASTRAGALUS*
PLANTS IN THE ARID WESTERN U.S.

A Dissertation

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Joseph M. Statwick

June 2016

Advisor: Anna A. Sher

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Author: Joseph M. Statwick
Title: THE ECOLOGY AND EVOLUTION OF RARE, SOIL SPECIALIST
ASTRAGALUS PLANTS IN THE ARID WESTERN U.S.
Advisor: Anna A. Sher
Degree Date: June 2016

ABSTRACT

Organisms that specialize in uncommon habitats are, by their very nature, inherently uncommon. Specialization has its advantages, namely reduced competition and predation, but it also incurs costs. Specialists often have small population sizes, narrow ranges, and fragmented habitat, all of which engender negative consequences on an evolutionary timescale. Herein, I examine benefits and costs of specialization in selenium-hyperaccumulating plants in the genus *Astragalus* (Fabaceae). These plants are disproportionately likely to be rare and of conservation concern. Thus, I optimized germination pretreatments for *Astragalus* species such that seed loss can be minimized during *ex situ* cultivation, and found that physical scarification is most effective in breaking hard-seed dormancy. Through analysis of soil in seleniferous habitats, I found that soil hydrology can rapidly deplete bioavailable selenium, potentially further reducing the habitat available for accumulators. To better understand the relationship between soil bioavailable selenium and plant performance, I subjected *Astragalus* species to a gradient of selenium concentrations in the greenhouse. Both non-accumulators and hyperaccumulators had less herbivory with increasing selenium concentrations, and also grew larger, despite the energetic cost of selenium uptake. One potential explanation for their larger growth is that selenium reduced inadvertent drought stress during the experiment, so I tested that hypothesis using a full factorial experiment of drought stress

and selenium dosage. Although drought stress reduced lifespan and selenium extended it, there was no evidence that selenium ameliorated drought stress. As a case study of the potential population genetic consequences of specialization, I examined the genetic structure and diversity of two allopatric cryptic sister species of *Astragalus*. Despite known low pollen and seed dispersal and strong genetic isolation by distance, populations were relatively diverse and not substantially inbred. Additionally, the genetic data did not support a two-species arrangement, so I recommend the species be consolidated, although several populations are somewhat isolated and merit special conservation attention. In summary, hyperaccumulators derive ecological benefits from their specialization that outweigh its metabolic cost, but may suffer low connectivity between populations, if not necessarily inbreeding depression. Conservation efforts should thus focus primarily on minimizing threats to and preserving connectivity of specialist habitats.

ACKNOWLEDGEMENTS

I would like to thank my committee: Anna Sher, Jennifer Ramp Neale, Scott Nichols, and Robin Tinghetella, as well as my committee chair, Michael Daniels. I would also like to acknowledge the former members of my committee, Martin Quigley and Tom Quinn. I thank my additional co-authors, Brian Majestic and Anna Williams; the members of the Denver Botanic Gardens Research and Conservation Department; and the DU Organismal Biologists group. I thank the journal editorial staffs and anonymous peer reviewers to whom I submitted these chapters and my additional research assistants Alex Dvorak, Graham Frank, Margarita Salazar, Jullie Ngo, Nathan Kingsley, Lisa Clark, Grace Johnson, and Christina Szabados. I thank the other contributors who provided advice, support, supplies, samples, permits, etc., including but not limited to Jennifer Ackerfield, Mary Barkworth, Paul Bedard, Scott Bingham, Ronald Bolander, Mike Bone, Benton Cartledge, Roger Clark, Carol Dawson, George Ferguson, Robert Fitts, Jill Handwerk, Anna Lincoln, Sarah Lindsey, Mitchell McGlaughlin, Ernie Nelson, Michael Piep, Elizabeth Pilon-Smits, Dana Truman, Stanley Welsh, and Katy Wilcox Wieczorek. This work was supported by funding from the University of Denver Graduate Studies Doctoral Fellowship, Pustmeuller Fellowship, Summer Undergraduate Fellowship, and Graduate Studies Dissertation Fellowship, and also by Denver Botanic Gardens, the Bureau of Land Management, the Colorado Native Plant Society, and the Colorado Mountain Club. Finally, I would like to thank my family and friends, particularly Malee Statwick, Patricia Statwick, James Statwick, Barbara Alexander, and Somkit Rasameetham, for their personal and professional support.

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CHAPTER ONE: GERMINATION PRETREATMENTS TO BREAK HARD- SEED DORMANCY IN *ASTRAGALUS* (FABACEAE)

Summary

Conservationists often propagate rare species to improve their long-term population viability. However, seed dormancy can make propagation efforts challenging by substantially lowering seed germination. Here I statistically compare several pretreatment options for seeds of *Astragalus cicer*: unscarified controls and scarification via physical damage, hot water, fire, acid, and hydrogen peroxide. Although only 30% of unscarified seeds germinated, just physical scarification significantly improved germination, whereas two treatments, hot water and fire, resulted in no germination at all. I recommend that rare species of *Astragalus*, as well as other hard-seeded legumes, be pretreated using physical scarification. Other methods have the potential to be effective, but may require considerable optimization, wasting precious time and seeds.

Introduction

Propagating wild species in greenhouses and common gardens for their restoration or reintroduction in native habitats can be an effective method of improving the size and viability of rare or threatened populations (Maunder, 1992; Menges, 2008). Such *in situ* and *ex situ* propagation techniques are beneficial, so long as these techniques

are successful in establishing additional reproductive adults in novel, degraded, or extirpated sites (Maunder, 1992; Menges, 2008). If, however, reintroduction is unsuccessful (which it usually is (Godefroid *et al.*, 2011)), it accomplishes nothing more than wasting resources and even further threatening the species by removing seeds that would have become the future seed bank.

At ~3270 species, *Astragalus* (Fabaceae) is the largest genus of flowering plants in the world (Watrous and Kane, 2011). Though a few *Astragalus* are weedy, wide-ranging generalists, specialization on uncommon and infertile soils seems to be a hallmark of the genus (Barneby, 1964). Unfortunately, this specialization appears to restrict many species to small geographic ranges, making them more vulnerable to extinction. In the United States alone, the US Fish and Wildlife service (2014) has listed 3 *Astragalus* species as under review, 5 as candidate, 5 as threatened, and 16 as endangered. Although the IUCN database (2014) contains less than one half of one percent of known *Astragalus* species, nearly 40 percent of those with sufficient data are considered “vulnerable” or worse (9 vulnerable, 12 endangered, 18 critically endangered, and 1 extinct). NatureServe (2014), meanwhile, lists 100 vulnerable, 58 imperiled, and 31 critically imperiled species, which combine to nearly a third of the 616 *Astragalus* species in its database.

Astragalus species, like most temperate legumes, as well as species of as many as 15 different plant families, have hard seed coats and physical dormancy, which often require scarification or stratification to break (Baskin *et al.*, 2008; Long *et al.*, 2012). In particular, low germination rate is a known “weak point” in the life cycle of several rare species of *Astragalus*, including *A. nitidiflorus* (Vicente *et al.*, 2011), *A. bibullatus*

(Albrecht & Penzagos, 2012), and *A. arpilobus* (Long *et al.*, 2012). Although prolonged dormancy of the seed bank may contribute to the maintenance of genetic diversity in rare *Astragalus* such as *A. albens* (Neel, 2007) in the wild, this dormancy is counterproductive for propagation efforts.

Many scarification treatments have been explored in the literature, including dry heat (Albrecht & Penzagos, 2012; Chou *et al.*, 2012; Long *et al.*, 2012), wet heat (Long *et al.*, 2012), stratification (Acharya *et al.*, 2006; Albrecht & Penzagos 2012; Long *et al.*, 2012), physical scarification (Acharya *et al.*, 2006; Albrecht & Penzagos, 2012), acid (Acharya *et al.*, 2006; Long *et al.*, 2012) smoke water (Chou *et al.*, 2012), etc., but it is rare that the results of more than one or two treatments have been compared in the same study. Because different species and even collections within species vary in germination rate, (Acharya *et al.*, 2006; Albrecht & Penzagos, 2012), the results of these studies are not directly comparable to one another in order to determine the most effective scarification treatment. I therefore explored six different pre-planting seed treatments (e.g. chemical and physical scarification) to determine which would best promote germination in the generalist forage crop, *Astragalus cicer* “Oxley”.

Methods

Astragalus cicer (L.) (cicer milkvetch) is an old-world native that was introduced to North America as a hardy, palatable forage crop (Acharya *et al.*, 2006). “Oxley” is an ecotype that was first collected in the former USSR and introduced to the United States in 1971 (Acharya *et al.*, 2006). Although *A. cicer* is not rare, it is a suitable model for rare species because it is readily commercially available without threatening wild populations,

and because it, like its rare congeners, is well known for its slow stand establishment, largely due to low germination rates and prolonged seed dormancy (Acharya *et al.*, 2006).

I exposed 50 *A. cicer* seeds (Granite Seed, Denver, CO) to each of six different scarification treatments, starting March 15, 2013 at Denver Botanic Gardens (DBG) in Denver, Colorado. The scarification treatments were physical damage, hot water, hydrogen peroxide, acid, fire, and a control. Control seeds were planted in 1 cm² germination pots, without scarification, on the surface of a seed starter mix, and covered with approximately 3 mm of vermiculite. Treated seeds, except fire, were planted in the same manner, but after a scarification treatment. I physically scarified seeds by cracking the seed coat opposite the radicle with a pair of infant nail clippers, being careful to not damage the endosperm or embryo. For the hot water treatment, seeds were placed in a thermos and covered with boiling (~95 C) water. I closed the thermos and allowed the seeds to soak for 20 hours before planting. Peroxide seeds were soaked in pure ZeroTol (27% hydrogen peroxide) for one hour before planting. Acid treated seeds were soaked in lab grade sulfuric acid (98%) for five minutes. Fire treated seeds were scattered on the soil surface of two 10 cm clay pots, and then covered with ~2 cm of dry pine needles and grass. The dry material was lit with a butane torch and allowed to burn until naturally extinguished. Approximately 2 mm of ash remained, and the seeds were left undisturbed to germinate in the clay pots. The total number of seeds germinated in each treatment was recorded approximately twice per week for one month.

All seedlings were reared in a propagation greenhouse at DBG. The potting soil was checked daily and kept evenly moist by DBG horticulture staff. Plants were exposed

only to natural sunlight, which, given the date and latitude, ranged between approximately 12 hours at the beginning of the trial and 13 and a half hours at the end of the trial.

Germination data were analyzed with a proportional hazards analysis using JMP v10. This analysis type is well suited to germination data in that it is intended for time series datasets composed of binary data in which each observation is a replicate (i.e. each seed has germinated or not germinated), and compares observed and expected frequencies with a χ distribution. Repeated measures ANOVA was not used because calculating the variance of proportions based on grouped binary data is inappropriate in that the proportions are both ordinal and bounded between 0 and 1.

Results

Seed treatment was an exceptionally strong predictor of seed germination success ($\chi^2=101.4$, $P<0.0001$, $df=5$, $n=300$). Physically scarified seeds germinated most quickly, and were more than twice as successful as any other treatment (Table 1.1), with a final germination rate of 74% over 33 days (Figure 1.1). Statistically similar percentages of unscarified, acid scarified, and peroxide scarified seeds germinated (30%, 34%, and 26%, respectively) (Table 1.1). No seeds from either hot water or fire scarification treatments germinated. Across all treatments, the bulk of germination occurred within the first 2 weeks, with virtually no germination after that point (Figure 1.1).

Table 1.1: Pairwise risk ratios for treatments, expressed as the ratio of the germination success of the row relative to the column. n=50 for each treatment. * represents statistical significance at the $P < 0.001$ level.

Treatments	Control	Hot Water	Sulfuric Acid	Nail Clippers	Hydrogen Peroxide	Fire
Control	1	>100*	0.85	0.32*	1.17	>100*
Hot Water	<0.01*	1	<0.01*	<0.01*	<0.01*	1
Sulfuric Acid	1.17	>100*	1	0.37*	1.38	>100*
Nail Clippers	3.17*	>100*	2.69*	1	3.72*	>100*
Hydrogen Peroxide	0.85	>100*	0.72	0.27*	1	>100*
Fire	<0.01*	1	<0.01*	<0.01*	<0.01*	1

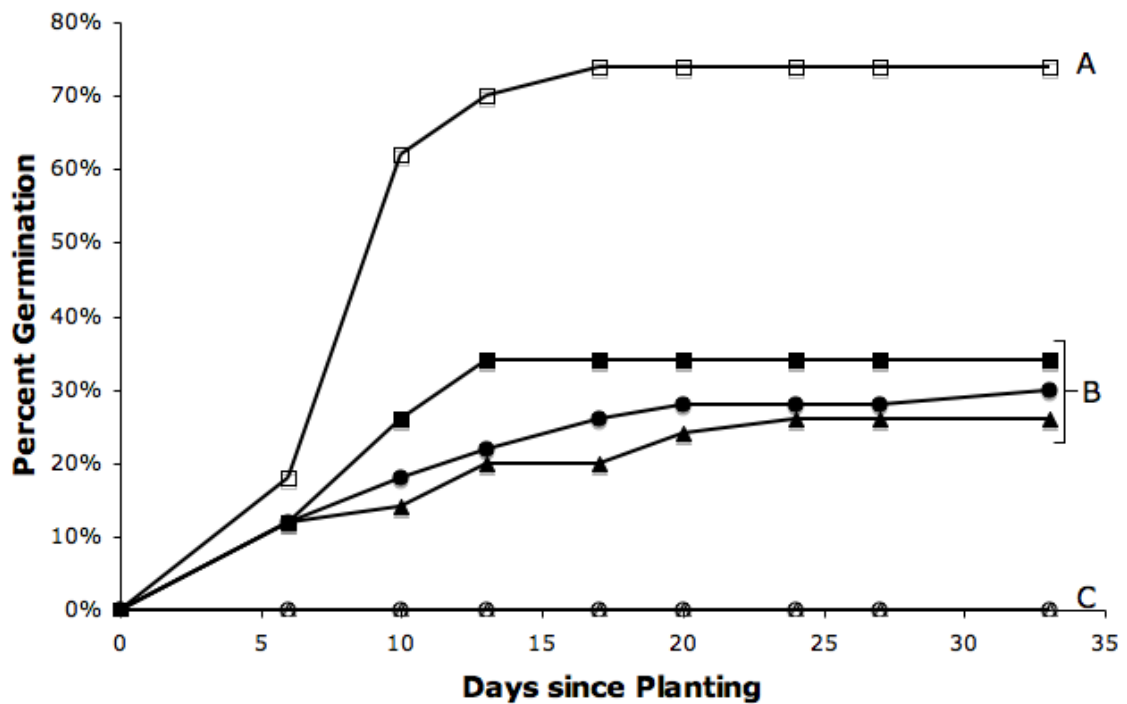


Figure 1.1: Germination rates over time for different scarification treatments for *Astragalus cicer*. The treatments include an unscarified control (closed circles) and seeds scarified with hot water (open circles), sulfuric acid (closed squares), nail clippers (open squares), hydrogen peroxide (closed triangles), and fire (open triangles). Letters indicate statistically different treatments via proportional hazards analysis.

Discussion

Although many scarification treatments have been attempted for *Astragalus* species, my data show that not all treatments are equal in efficacy. In fact, only one treatment, physical scarification, was significantly better than the control, and both the fire and hot water treatments were significantly worse than the control, effectively sterilizing all of the seeds.

Based on my data, I recommend that propagation efforts involving *Astragalus* species use physical scarification as the primary method for breaking seed dormancy. Whereas other scarification treatments have been effective in certain circumstances, physical scarification has generally been shown to be the most effective treatment in studies that have compared it to alternative methods (Acharya *et al.*, 2006; Albrecht & Penzagos, 2012). The only downside to physical scarification, the labor-intensive nature of damaging the seed coat with sandpaper, a razor blade, or nail clippers, can be overcome with commercial equipment, if necessary, although at the cost of slightly higher seed loss to excessive damage (Acharya *et al.*, 2006).

Whereas other studies have demonstrated that methods involving cold, heat, acid, etc., can improve germination over controls, I recommend against their use in *Astragalus*, as the studies comparing different durations and intensities (temperature, concentration) of these treatments have found a relatively narrow range of optimal conditions (Albrecht & Penzagos, 2012; Chou *et al.*, 2012; Long *et al.*, 2012). Treatments of insufficient duration or intensity appear to be incapable of breaking seed dormancy, whereas treatments of excessive duration or intensity damage not only the seed coat, but the

embryo as well, causing a loss of viability (Albrecht & Penzagos, 2012; Chou *et al.*, 2012; Long *et al.*, 2012). This is evidenced in my own study by the apparently insufficient acid and peroxide treatments compared to the apparently excessive fire and hot water treatments. I feel that, particularly for rare species for which seeds are limited, attempting to optimize these techniques for each species is an unnecessary waste of resources when physical scarification is equally if not more effective.

CHAPTER TWO: SOIL SELENIUM IN SELENIFEROUS ENVIRONMENTS OF THE ARID WEST

Summary

Seleniferous soils are host to a diverse and unique community of plants, animals, and microorganisms. Often, studies of these organisms, if they report selenium at all, only report the total selenium content of the soil. We conducted a field survey of soils to determine a) whether total selenium is a reliable proxy for bioavailable selenium, and b) the general characteristics of typical seleniferous soils. We analyzed soils from 32 seleniferous and nearby non-seleniferous habitats across western Colorado. In normal, low-selenium soils, the relationship between total and bioavailable selenium is roughly linear. In seleniferous soils however (total Se >2mg/kg), there is no relationship between total and bioavailable selenium. Also, these soils can be broadly characterized by two principal axes: a metals-rich axis likely explained by the mineralogy and depositional environment of the parent rock, and a soluble, salt-rich axis likely explained by soil weathering and hydrology. There is considerably more variation along the former axis, but selenium content, particularly bioavailable selenium, is influenced by the latter. However, parent rock mineralogy, particularly phosphorus content, seems to drive soil organic matter, implying a primarily phosphorus limited ecosystem.

Introduction

Selenium is both an essential nutrient and an acute toxin and environmental pollutant. In the arid west, selenium generally occurs in two forms, the non-available elemental form, and the highly bioavailable selenate form (Oldfield, 2002). One might expect that biotic and abiotic processes would keep these two forms in dynamic equilibrium, and so many studies of organisms from seleniferous ecosystems (if they report soil selenium at all – some do not (e.g. Cowgill and Landenberger, 1992; Somer and Çaliskan, 2007; Galeas et al., 2008)) only report total soil selenium or are ambiguous about whether they are reporting total or bioavailable selenium (e.g. Galeas et al., 2007; Freeman et al., 2009; Sors et al., 2009).

Seleniferous soils in the western United States have been of scientific interest at least since the 1890s, when researchers were trying to determine the cause of a mysterious illness affecting grazing cattle, known at the time as “alkali disease” (Trelease, 1942). Also called “blind staggers”, the disease caused gastrointestinal pain, listlessness, aimless wandering, paralysis, and death. It was eventually discovered that certain plants growing on seleniferous soils were also very high in selenium, and could cause the disease if fed to cattle in controlled settings (Beath et al., 1934). Feeding a sheep with as little as 1.3 g/kg of these plants was sufficient to cause death in just a few hours (Beath et al., 1934). These “indicator plants” were frequently found to have more than 1000mg/kg selenium in their aboveground tissues, and were noted to be indicative of seleniferous sedimentary formations (Trelease and Trelease, 1937; Beath et al., 1939a). While normal soils can generally contain less than 2mg/kg of selenium (Mayland et al.,

1989; Oldfield, 2002) these seleniferous strata often contain more than 10 mg/kg of selenium, and have been reported to contain up to 1200 mg/kg in rare instances (Mayland et al., 1989).

Some of the plants that inhabit these soils are now referred to as hyperaccumulators, because of their ability to take up trace elements at hundreds or thousands of times background levels, without apparent harm (Brooks et al., 1977, Boyd, 2007). The most well known selenium accumulators are in the genera *Astragalus* (Fabaceae) and *Stanleya* (Brassicaceae) (Freeman et al., 2006; van der Ent et al., 2013), although at least 20 taxa from 7 families have been demonstrated to hyperaccumulate the element (Krämer, 2010). However, even in the state of Colorado alone, many of the known selenium hyperaccumulators are tracked as rare or threatened, including *Astragalus debequaeus*, *A. eastwoodiae*, *A. linifolius*, *A. nelsonianus*, *A. oocalycis*, *A. osterhoutii*, and *A. rafaensis* (CNHP, 1997+). This is due in part to habitat degradation from uranium and natural gas extraction, because these resources often coincide with seleniferous strata (Beath, 1943; Presser, 1994). In fact, one of the collections in this survey was at a former population of *A. debequaeus* that had been extirpated by a well pad, and another was collected from the disturbed soil covering a recently buried pipeline. However, the rarity of these species is most likely primarily attributable to the limited extent and discontinuous nature of seleniferous soils to begin with. Thus, our understanding of the form and distribution of seleniferous soils can help inform the conservation of these species and their ecological partners.

Selenium, like its elemental neighbor, sulfur, is quite volatile in its liquid and gaseous forms, and thus tends to be reduced in intrusive igneous rocks, and marginally enriched in extrusive rocks, particularly basalt, ash, and other ejecta (Malisa, 2001). However, because selenium strongly adsorbs to clay minerals and may be bioenriched by aquatic organisms, it is typically found at some of its highest levels in clay-rich sedimentary formations, including mudstones and shales - particularly those that were deposited during periods with high levels of volcanism (Byers et al., 1936; Beath et al., 1939a; Mayland et al., 1989). Such conditions were not uncommon during the Cretaceous and early Paleogene periods, when the Sevier and Laramide orogenies caused substantial volcanism in the western US, and the Western Interior Seaway covered much of what is now the Rocky Mountains, creating an ideal depositional environment for mudstones and shales. Indeed, many Cretaceous and Paleocene sediments of the western US are dangerously enriched in selenium content (Beath et al., 1939a,b; Kulp and Pratt, 2004)

However, mineralogy is not the only factor affecting selenium levels in the environment. The ionic forms of selenium in particular are highly soluble, and can be easily leached from or deposited in soils via precipitation and hydraulic conductance (Kulp and Pratt, 2004; Tuttle et al., 2014b). The southeastern United States, for example, has soils that are generally deficient in selenium, due to high levels of rainfall and leaching (Mayland et al., 1989). Likewise, precipitation, among other factors, causes parts of the Tibetan Plateau to have soil so deficient in selenium that it causes chronic nutrient deficiency in humans who live in the area (Wang et al., 2013). On the other hand, in Hawaii, precipitation is positively associated with selenium content, possibly because

rainwater deposits atmospheric selenium from the islands' volcanic emissions onto the iron-rich lava stone, where it complexes into highly insoluble ferric selenite and is sequestered (Byers et al., 1936).

Because the biotic and abiotic cycling of selenium may disproportionately deplete or enrich only certain chemical forms of the element, the ratio between total and bioavailable selenium may not be predictable. If that ratio is not consistent, researchers could potentially mischaracterize seleniferous and non-seleniferous areas by conflating the two. Herein we tested the implicit hypothesis that total selenium is a reasonable proxy for bioavailable selenium. We also sought to better characterize the soils of seleniferous habitats in order to improve the success of biodiversity conservation efforts in these areas.

Methods

Soil Collection

We collected soil samples from 32 sites across western Colorado, where selenium hyperaccumulators in the genus *Astragalus* occur in close proximity to congeneric non-accumulators based on protected occurrence data from the Colorado Natural Heritage Program. Our collections were taken in two primary areas: in and around the town of DeBeque, and along seleniferous formations ringing the Uncompahgre Plateau. Seleniferous collections near DeBeque were largely soils derived from the Atwell Gulch member of the Wasatch Formation. The Atwell Gulch member is a known seleniferous stratum that is mud-dominated and straddles the Paleocene-Eocene boundary. (Beath et

al., 1939a,b). Seleniferous collections around the Uncompahgre Plateau were primarily from soils derived from the Morrison formation, another known seleniferous stratum, which was deposited during the late Cretaceous (Beath et al., 1939a,b). Although the formation is broadly seleniferous, the Salt Wash member is particularly so (Beath, 1943). Non-seleniferous collections were of soils derived from neighboring strata, which have similar geologic, pedologic, and climatological characteristics (Figure 2.1).

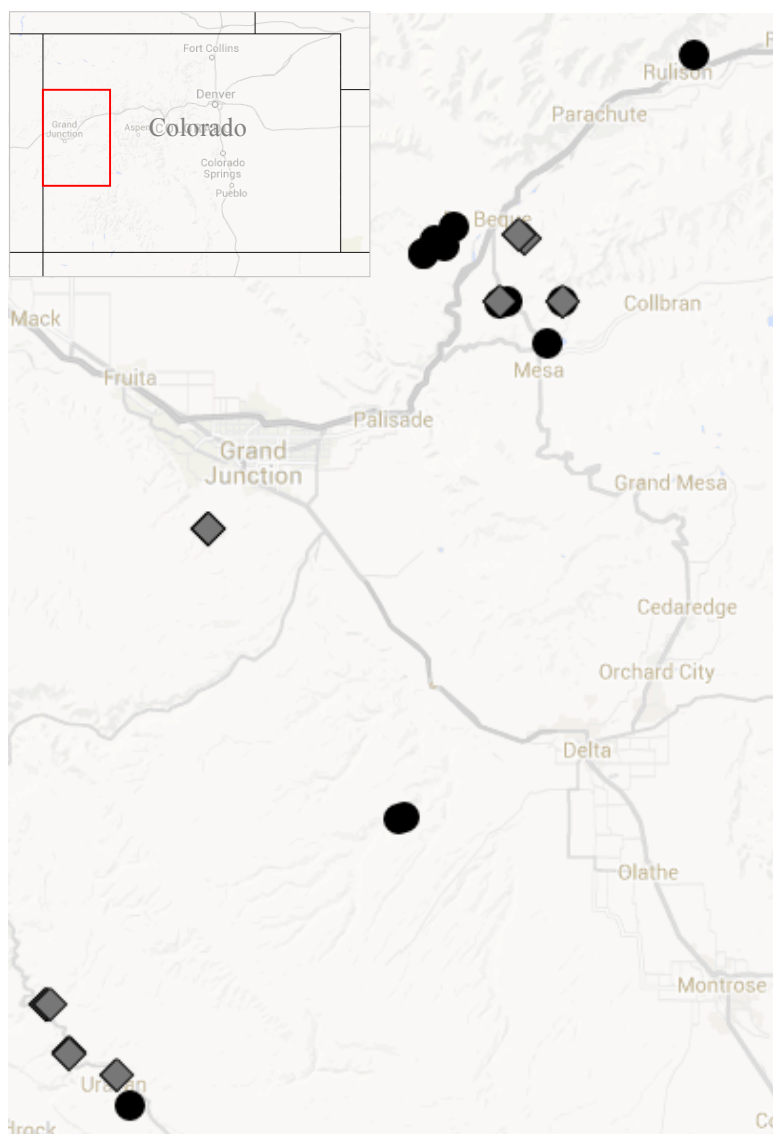


Figure 2.1: Map of soil collection sites in Western Colorado (inset). Sites were chosen for having seleniferous plants growing in close proximity to non-seleniferous plants. Sites which had high total selenium ($>2\text{mg/kg}$) are depicted as grey diamonds, while those with low total selenium ($<2\text{mg/kg}$) are depicted as black circles.

Soil Analysis

Soils were analyzed by the Soil, Water and Plant Testing Laboratory at Colorado State University. Bioavailable essential nutrients were determined via AB-DTPA extraction. Total and plant available selenium were determined with ICP-AES. Site

precipitation data were taken from the PRISM climate group 30-year normals (1981-2010), based on the 800m spatial resolution (<http://www.prism.oregonstate.edu/>).

Statistical Analysis

All data sets were analyzed via JMP Pro v11. We tested the relationship between total Se and bioavailable Se using both log-transformed and untransformed data using standard linear regression. In order to characterize the general properties of the soils we collected, we performed several exploratory univariate and multivariate analyses. The relationship between soil texture and lime content was tested via chi square test. For other analyses of soil texture or lime content, the variables were assigned arbitrary ordinal numerals and analyzed non-parametrically. Soil textures were ranked in order of clay content (sandy clay loam = 1, sandy clay = 2, clay = 3), and lime content was ranked in order of increasing lime (low = 1, medium = 2, high = 3, very high = 4). For univariate comparisons of either clay or lime and another soil variable, we used the non-parametric rank-order Spearman's rho test. We used untransformed and rank-order data for our PCA, as it does not assume normality.

Results

The ability of total selenium to predict bioavailable selenium was mixed (Figure 2.2, Figure S2.1). In normal soils (<2mg/kg total Se), log-transformed total selenium was a strong predictor of log-transformed bioavailable selenium ($P < 0.001$, $n=16$, $r^2_{adj}=0.52$). Even still, the bioavailable portion averaged 35% of total selenium, but

ranged between 0.1% and 82%. In seleniferous soils ($>2\text{mg/kg}$ total Se), there was no relationship between log-transformed total and log-transformed bioavailable selenium ($P=0.65$, $n=16$, $r^2 \text{ adj.}=-0.05$). Untransformed data produced qualitatively similar results for both normal soils ($P<0.01$, $n=16$, $r^2 \text{ adj.}=0.46$) and seleniferous soils ($P=0.35$, $n=16$, $r^2 \text{ adj.}=-0.01$) (Figure S2.1). Soil texture was not a significant predictor of total or bioavailable selenium (Spearman $\rho = -0.27$, $P=0.13$; Spearman $\rho = -0.18$, $P=0.32$; respectively)

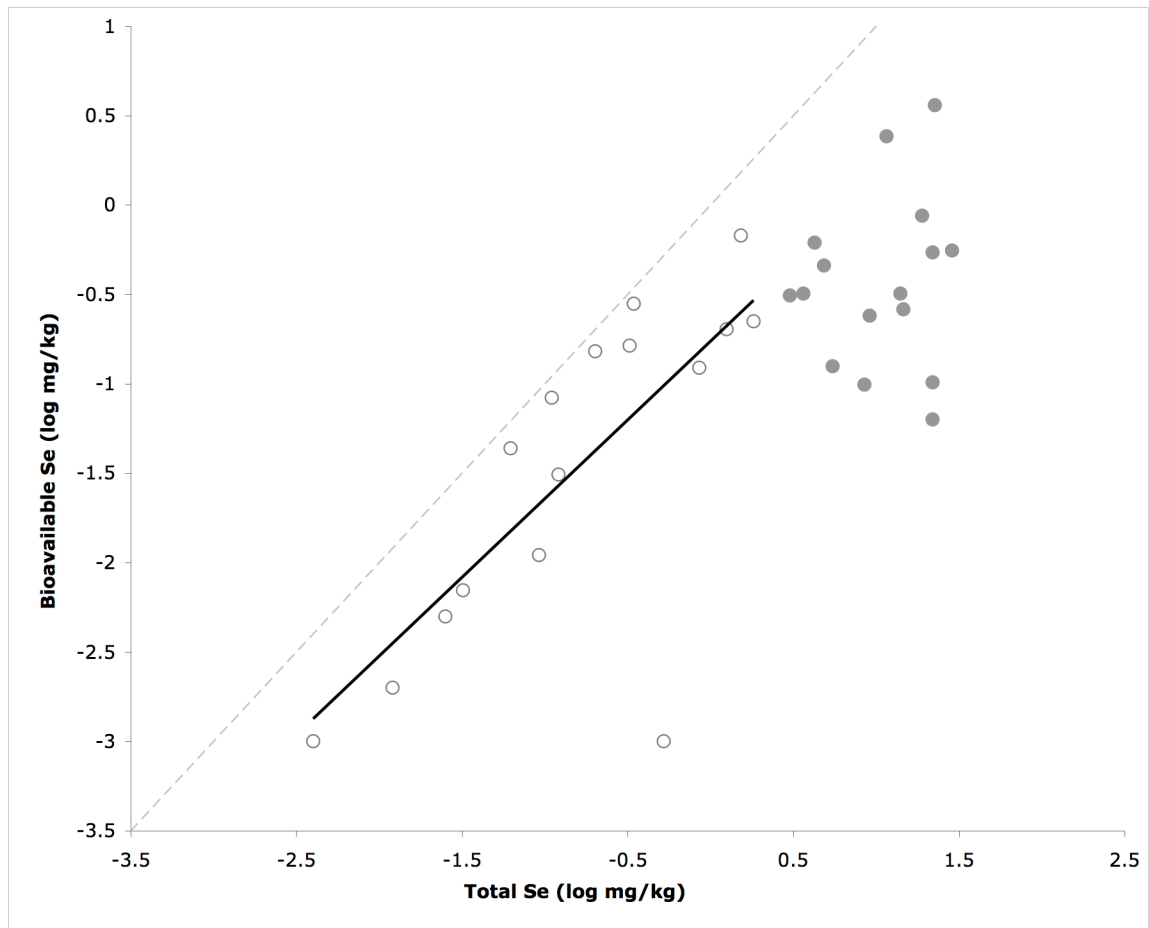


Figure 2.2: Log-transformed total soil selenium versus log-transformed bioavailable soil selenium. Unfilled circles represent normal soils, i.e. those with $<2\text{mg/kg}$ total Se. The solid line represents the best fit line for normal soils. The filled circles represent seleniferous soils ($>2\text{mg/kg}$ total Se). There is no trendline for seleniferous soils because the relationship is not significant. The dashed light grey line represents the identity line, i.e. a 1:1 bioavailable fraction.

A PCA of continuous soil variables revealed two main groups of vectors (Figure 2.3). The first grouping, which is largely oriented along PC1, contains vectors for the metals copper, zinc, and iron, as well as for sandy texture, phosphorus, and % organic matter. Mean annual precipitation, when included, aligns with PC1, but the eigenvector loading was very weak, so we excluded it from the analysis, and its inclusion or exclusion does not affect the loading of other variables. The second grouping is

orthogonal to the first, and is largely oriented along PC2. It contains the vectors for nitrate, bioavailable selenium, potassium, and conductivity. Manganese, total Se, lime, and pH are intermediate between these axes. Manganese is positively correlated with both sets of vectors, while lime, pH, and total Se are positive for the second group of vectors, but negative for the first (Figure 2.3a). If we exclude non-seleniferous soils (<2mg/kg total Se) from the analysis, we see nearly identical results (Figure 2.3b).

Despite the differences, all of the soils collected had some broad similarities (Table 2.1). All were shallow (sometimes <10cm to bedrock), dry, azonal, and rocky, and are most likely a combination of orthents and argids. They appeared to be mostly composed of recently eroded parent rock, as they were generally collected on or near talus slopes, alluvial washes, or braided arroyos. Sand and clay dominated the samples, with 53% of samples being sandy clay loam, 28% being sandy clay, and 18% being clay. Soil texture was not statistically associated with lime content ($\chi^2=0.38$, $n=32$, $df=6$). Lime content ranged from low to very high (with a majority of samples being very high), and was moderately associated with pH, with more lime leading to a more basic soil (Spearman $\rho = 0.40$, $P<0.05$). Soil texture was also associated with pH, with more clay-rich soils being more basic (Spearman $\rho = 0.61$, $P<0.001$). The pH range was approximately normally distributed, from weakly acidic to weakly basic, with a mean of 7.65. Organic matter content was very low in all samples, with a mean of 0.65% and a maximum of just 1.2%.

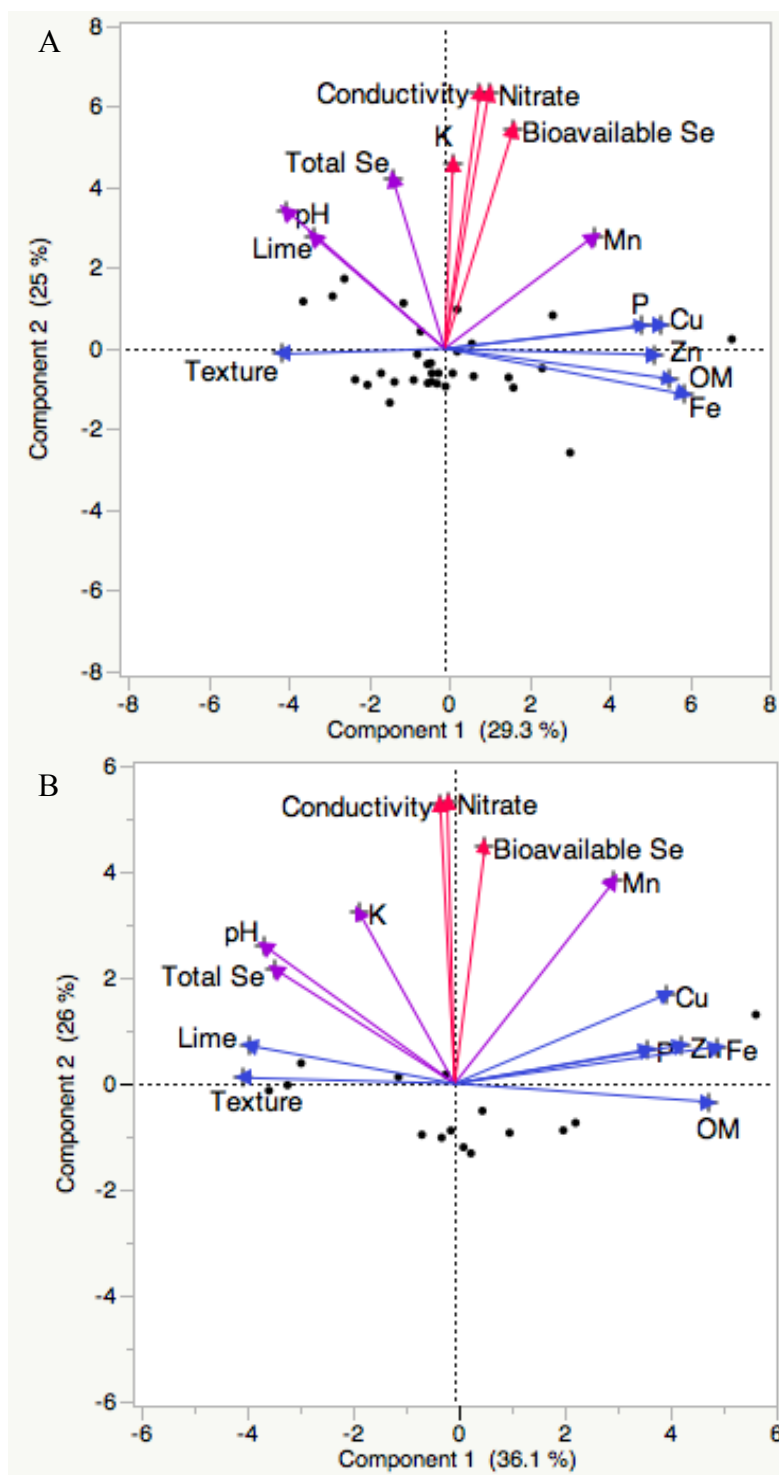


Figure 2.3: PCA biplot of soil variables for all soils collected (A) and only seleniferous soils (>2 mg/kg total Se) (B). Blue vectors are those which orient primarily along PC1, red vectors align primarily along PC2, and purple vectors are intermediate between the two axes.

Table 2.1: Table of average soil variables across all 32 sites. Most variables were substantially right skewed, so we report both mean and median below. Conductivity is reported in mmhos/cm. All nutrients are reported as mg/kg. Annual precipitation data were estimated with the PRISM climate model, and are reported in mm.

Variable	Mean \pm SD	Median	Interquartile range
pH	7.65 \pm 0.47	7.65	7.40-7.88
Conductivity	2.78 \pm 12.18	0.40	0.30-0.70
% Organic	0.65 \pm 0.25	0.60	0.43-0.80
Nitrate	8.38 \pm 32.69	1.95	1.13-2.92
Phosphorus	1.65 \pm 2.15	1.00	0.40-1.85
Potassium	243.4 \pm 109.6	208.0	168.0-303.8
Zinc	0.420 \pm 0.495	0.314	0.240-0.422
Iron	3.52 \pm 1.61	3.35	2.68-3.98
Manganese	2.82 \pm 1.45	2.47	1.73-3.17
Copper	1.80 \pm 1.04	1.65	1.34-2.15
Total Selenium	6.94 \pm 8.77	2.41	0.14-13.38
Bioavailable Selenium	0.40 \pm 0.74	0.18	0.05-0.42
Annual Precipitation	332.9 \pm 32.3	328.6	308.2-345.5

Discussion

It is clear from our results that while total selenium may be a fairly reasonable proxy for bioavailable selenium in “normal” soils (although it only explains ~50% of the variance), it is an incredibly poor proxy for bioavailable selenium in geologically seleniferous soils. Under ideal conditions, plants (both hyperaccumulators and non-accumulators) take up selenium roughly proportionally to bioavailable supply, although this proportion can vary tremendously between species or even within species under different climatic and ecological conditions (Wang et al., 2013; Statwick et al., 2016). Studies of seleniferous habitats should take care to report bioavailable selenium content, not necessarily total selenium. Inferences about plant behavior or plant communities made from total selenium alone may lead to erroneous conclusions. Also, although

selenium is known to adsorb strongly to the clay fraction in soils (Goldberg, 2014), we found no significant relationship between estimated clay content and either total or bioavailable selenium, meaning that this relationship is likely much more dynamic in field settings.

Based on our PCA, the seleniferous soils of the western slope of Colorado can be described by two major axes (Figure 2.3). We suggest the first of these axes largely represents the depositional environment and mineralogy of the parent rock. Soils more positive in PC1 are sandier, lower in lime, more acidic, and higher in available mafic metals. Since mafic metals, particularly iron, are also associated with higher phosphorus content in rocks (Porder et al., 2012), we were not surprised to see phosphorus also align with PC1. The combination of larger grain size (implying shorter transport distance and/or higher turbidity), less lime (from deepwater organisms), and more metals (from nearby volcanic sources) leads us to speculate that soils that are more positive along PC1 are derived from sedimentary rocks that were originally deposited terrestrially or in relatively shallow water, perhaps during periods of sea level regression or on topographically higher areas (Sloijes et al., 2008). Soils that are negative on PC1 are thus perhaps derived from sediments deposited in deeper water, during periods of transgression, or in topographically lower areas. While it is possible that PC1 describes quaternary weathering and transport, we think this is unlikely for several reasons. Firstly, if PC1 described weathering post-lithification, we would expect lime content to be positively correlated with both grain size and metal content (all three would weather together), not negatively. Second, because soils were generally collected immediately

downslope from their parent rocks and in an arid climate, we expect quaternary weathering of more resistant metaliferous minerals to be minimal.

PC2, on the other hand, is made up of vectors representing highly soluble compounds like selenate, nitrate, potassium, and others that influence conductivity, so it likely describes soil hydrology. Soils that are highly positive on PC2 may be those in shallow, depressed, or poorly drained areas in which evaporation is dominant, and soluble compounds are concentrated near the surface. Soils that are highly negative along PC2 are those in which leeching is dominant, and soluble compounds are removed from the soil. Soluble selenium ions are very rapidly depleted by precipitation, especially in arid regions or young soils, where the high efflux of selenium into surface and groundwater can be acutely toxic (Presser, 1994; Kulp and Pratt, 2004; Kuisi and Abdel-Fattah, 2010; Mast et al., 2014; Tabelin et al., 2014a,b; Tuttle et al., 2014a,b; Tamoto et al., 2015), so we were not surprised to find bioavailable selenium aligned strongly with PC2. It is worth noting that although total selenium seemed to be marginally influenced by both axes, bioavailable selenium was entirely unrelated to the mineralogy of the parent rock, and influenced solely by hydrology.

While one might expect phosphorus to align with PC2, phosphorus is relatively insoluble, and soils within a given climatic region have been shown to have a phosphorus level that correlates much more strongly with parent rock composition than with soil weathering (Porder et al., 2012). Weathering eventually does deplete soil phosphorus, but only substantially so on the time span hundreds of thousands of years (Porder et al, 2012; Newman and Hart, 2015).

We admit that it seems counterintuitive for organic matter to be loaded on PC1 rather than PC2. Given a median phosphorus concentration of 1 mg/kg, this ecosystem appears to be primarily phosphorus limited. This is surprising given that these are young soils and therefore expected to be nitrogen limited, not phosphorus limited (Walker and Syers, 1976; Newman and Hart, 2015). However, young soils can be phosphorus limited if 1) the parent material is already low in phosphorus, 2) low weathering rates limit available phosphorus inputs, and/or 3) high nitrogen inputs from soil fixation or aeolian deposition substantially increase the soil N:P ratio (Menge et al., 2012). The first of these conditions (low parent rock phosphorus) is likely met in this system, because the median phosphorus content of carbonate rocks (290 mg/kg) and sandstones (500 mg/kg) is much lower than that of the igneous rocks (900-1000 mg/kg) that form the basis of the classical model (Porder et al., 2012). The second of these conditions (low weathering rates) is likely also met, because of the arid climate. The third condition (high N inputs) is perhaps unlikely given a median 2mg/kg of nitrate, but nitrate flux may still be high because of increasing anthropogenic atmospheric nitrate deposition in the arid west (Fenn et al., 2012), and because *Astragalus* hyperaccumulators are nitrogen fixing legumes (Alford et al., 2012). Thus, it seems reasonable to conclude that this particular ecosystem is predominantly phosphorus limited, rather than nitrogen limited.

In short, the seleniferous soils of western Colorado are relatively inhospitable, even without taking the toxic levels of selenium into account. Their phosphorus limitation, low moisture, low organic matter, and lack of developed structure make them challenging for all but the most well adapted plants to survive. Despite seleniferous

communities generally being associated with seleniferous strata, the amount of bioavailable selenium in those soils can range from toxic to nearly deficient. Selenium availability is dictated in large part by hydrology, meaning that soils high in bioavailable selenium may also be saline or nitrate enriched, possibly making them more susceptible to desertification or exotic species invasion (Dukes and Mooney, 1999; Singh, 2009). Since these habitats are already limited in extent and many seleniferous species are obligate endemics, seleniferous communities might be especially prone to extirpation or extinction.

CHAPTER THREE: CHARACTERIZATION AND BENEFITS OF SELENIUM UPTAKE BY AN *ASTRAGALUS* HYPERACCUMULATOR AND A NON- ACCUMULATOR

Summary

Background and Aims: We characterized the relationship between soil and leaf concentrations of selenium in a hyperaccumulator and a non-accumulator to test the hypothesis that hyperaccumulators take up selenium while non-accumulators exclude it. We examined plant performance metrics and the ability of selenium to protect against herbivory by spider mites.

Methods: Known hyperaccumulator and non-accumulator species within the genus *Astragalus* were grown under a range of selenium concentrations and measured for tissue selenium, extent of herbivory, and vigor.

Results: Both hyperaccumulators and non-accumulators either failed to meet even the lenient threshold or exceeded even the strict threshold for hyperaccumulation depending on soil concentration. Both had decreased herbivory with increasing leaf selenium, and both grew larger at higher levels of selenium regardless of herbivory, despite a negative impact of higher relative uptake.

Conclusions: The relationships between selenium dosage and tissue concentrations matched only some model predictions. Under these conditions, the

bioconcentration factor was a better delimiter between species than the absolute tissue concentration. We provide evidence that despite the apparent cost of uptake, selenium can enhance the growth of hyperaccumulators even when herbivory is not a significant factor. We propose the term “elemental stimulation” for this phenomenon.

Introduction

Hyperaccumulators are plants that take up metals or other trace elements from the soil and concentrate them in aboveground tissues at hundreds or thousands of times background levels. Hyperaccumulation as a phenomenon has been recognized for eight decades (e.g. Beath *et al.*, 1934), even though the term was not coined until much later (see Brooks *et al.*, 1977). Hyperaccumulation is widespread in terms of the number of taxa that accumulate, the life histories of accumulators, and the variety of elements that are accumulated (Kramer, 2010; van der Ent *et al.*, 2013; Pollard *et al.*, 2014), and its study has broad phytotechnical (Barillas *et al.*, 2011), ecological (Boyd and Martens, 1998; Maestri *et al.*, 2010), and evolutionary (Broadley *et al.*, 2001) implications.

Tissue concentration thresholds to determine whether a species is a hyperaccumulator have been established for a range of elements. For the element selenium, plants are considered hyperaccumulators if, when grown on native soil, their leaves contain more than 1000mg/kg selenium dry weight (Boyd, 2007), although some authors argue that the threshold should be as low as 100mg/kg (van der Ent *et al.*, 2013). Determining whether a plant actually meets those criteria though is more complicated than it may appear (Boyd, 2007; Rascio and Navari-Izzo, 2011). *Astragalus bisulcatus*, a

widely studied, obligate selenium hyperaccumulator known to accumulate more than 10,000mg/kg (Shrift, 1969, Sors *et al.*, 2009), has been collected from its native habitat with tissue concentrations as low as 10mg/kg, with the median concentration being less than 300mg/kg (Shrift, 1969). A more recent study had similar results, with leaf concentrations at field sites ranging from 95 to just 160mg/kg (Sors *et al.*, 2009), far below the 1000mg/kg hyperaccumulator threshold, and with two of three sites containing no individuals above the 100mg/kg threshold.

The huge range in observed concentrations may be due to individual variation, but it is likely due to variation in soil as well. Native soil concentrations of selenium are far from uniform, with reports from “seleniferous soils” ranging from near zero up to 212mg/kg (Beath *et al.*, 1937). Most “normal” soils, meanwhile, contain less than 2mg/kg (Oldfield, 2002). It is also often unclear how much selenium in soil is bioavailable, given that there is a poor correlation between total and bioavailable selenium, with the bioavailable portion ranging at least from 0.2% to 81% of total soil selenium (Statwick, unpublished data).

A conceptual model of hyperaccumulation predicts that hyperaccumulators should increase tissue concentrations as a function of soil availability until they plateau due to saturation of the uptake pathways, negative feedbacks, or both. Non-accumulators are not expected to take up selenium at all until a concentration that results in rapid toxicity and death (van der Ent *et al.*, 2013). While these model predictions are intended to describe the metal uptake of plants in the field, the degree to which these uptake rates are actually a reflection of species’ abilities and not other factors is difficult to verify in an

uncontrolled, observational field setting. Climate (Bahtia et al., 2005), soil chemistry (Cakmak, 2007), rhizosphere (Lindblom et al., 2013), local genotypes (Roosens et al., 2003), and other factors can all affect the uptake of metals, and could all potentially covary with available soil metal content, thus making it nearly impossible to determine uptake in the field as a function of availability alone. Perhaps more importantly, hyperaccumulators and non-accumulators generally have little or no overlap in natural habitat, making paired comparisons impossible in the field. Thus, to determine the extent to which the relationship between availability and uptake is due to species capacity and not other factors, we compared the response of a congeneric hyperaccumulator and non-accumulator to a range of selenium concentrations that could be encountered by a plant in the field, using a greenhouse-based dose response design that allowed us to hold other variables such as soil texture and moisture constant. We chose to use spiked potting soil instead of field-collected soils in order to a) control for soil variability and b) avoid depleting the finite amount of selenium in a potted volume of field soil (Goolsby and Mason, 2015). Given the model above, we expected our hyperaccumulator to rapidly accumulate selenium at low soil concentrations, but to plateau at higher concentrations. We predicted that our non-accumulator would act as a “normal” plant, maintaining a constant low tissue concentration of selenium until some threshold, followed by rapid toxicity and death.

We also tracked plant performance metrics to investigate the hypothesis that selenium, although non-essential to plants (Novoselov *et al.*, 2002; Fu *et al.*, 2002; Lobanov *et al.*, 2009) and easily toxic (Brown and Shrift, 1982), can actually enhance the

growth of hyperaccumulators, even at extreme concentrations, and even in the absence of ecological stressors. Early literature includes both support (Trelease and Trellease, 1938, 1939; Davis, 1972) and criticism (Broyer *et al.*, 1972a,b), of this hypothesis, but trace elements stimulating growth directly has not appeared in reviews of hypotheses for adaptive value of hyperaccumulation (e.g. Boyd and Martens, 1998; Rascio and Navari-Izzo, 2011) until very recently (e.g. Cappa and Pilon-Smits, 2014).

It is well established in the fields of pharmacology and toxicology that very low doses of toxic compounds can have stimulatory effects on organisms due to the overcompensation of bioprotective response pathways, in a phenomenon known as hormesis (Calabrese *et al.*, 2007; Mattson, 2008). These benefits are characteristically inversely U-shaped, with a narrow range of concentrations that are beneficial, followed by detrimental toxic effects (Stebbing, 1982; Calabrese and Baldwin, 2001). Many plants show this pattern, benefiting from fortification with small amounts of trace elements such as zinc and selenium, which become toxic at higher concentrations (Xue *et al.*, 2001; Cakmak, 2007; Yao *et al.* 2009). Nonetheless, while hormetic responses are adaptive, it is typically not the stressor itself that is beneficial, but rather the downstream cellular responses to that stressor (Mattson, 2008). This would imply that selection on or upregulation of hormetic pathways should improve stress tolerance generally, but not necessarily the response to an individual stressor specifically (Stebbing, 1982). Indeed, it is often the case that exposure to low levels of one stressor (e.g., heat) can reduce the damage done by an entirely different stressor (e.g., cyanide) (Mattson, 2008).

Yet, there is a growing body of evidence that hyperaccumulators show positive responses to specific toxic elements (rather than toxic elements generally) at concentrations far higher than those that stunt the growth of normal plants (Küpper *et al.*, 2001; El Mehdawi *et al.*, 2012; Ghasemi *et al.*, 2014; Pollard *et al.*, 2014; Kazemi-Dinan *et al.*, 2015). Hyperaccumulators also appear to benefit across ranges of concentrations that are far broader (e.g. >1000-5000fold (Küpper *et al.*, 2001; Pollard *et al.*, 2014)) than the 10-20fold range typical of a hormetic response (Calabrese *et al.*, 2007). This implies that the direct benefits of hyperaccumulation are mechanistically different from hormesis, and could conceivably be acted upon by selection in such a way as to promote or maintain hyperaccumulation through evolutionary time.

Of the more commonly explored adaptive reasons for hyperaccumulation, only “elemental defense”, or the idea that trace elements protect plants from natural enemies such as herbivores and pathogens, has been examined in any depth (Rascio and Navari-Izzo, 2011). Elemental defense has been well supported in a wide range of systems (for review, see Boyd, 2007), and has become the primary adaptive justification for hyperaccumulation. However, it is not implausible or even improbable that other evolutionary drivers, including a direct benefit of trace elements themselves, exist in concert with elemental defense (Trumble and Sorensen, 2008). If trace elements can indeed enhance the growth of hyperaccumulators, even in the absence of natural enemies, we would predict that *Astragalus* selenium hyperaccumulators would grow larger with increasing selenium while non-accumulators would be negatively impacted by selenium, as it is toxic to most organisms at relatively low concentrations.

We designed an experiment to test the effects of soil selenium in the absence of other environmental stress, however, during our treatment period and despite control efforts, there was an unplanned and persistent infestation of two-spotted spider mite (*Tetranychus urticae*), a generalist cell-disruptor herbivore. Although this confounded our original intent, it gave us the opportunity to investigate the relationship between tissue selenium concentration and herbivory, and allowed us to examine the impact of both elemental defense and selenium dosage on plant performance. Because selenium in *A. bisulcatus* has been previously shown to deter spider mites (Quinn et al., 2010), we predicted that selenium would reduce herbivory in hyperaccumulators, but that its concentration would be too low to protect non-accumulators.

Methods

Study Species

We chose two species of *Astragalus* to investigate the hyperaccumulation of selenium. This genus is often thought of as broadly seleniferous, in part because of a substantial but unknown number of species that exhibit at least mild selenium tolerance (Davis, 1972; Wang *et al.*, 1999; Moreno Rodriguez *et al.*, 2005; Sors *et al.*, 2009). In fact, there are only 25 known species (<1% of the genus) classified as true hyperaccumulators (Barneby, 1964; Welsh, 1985). This gives us the opportunity to test the response to selenium of congeneric species with different *a priori* tolerance. *Astragalus bisulcatus* (Hook) A. Gray is a fairly widespread hyperaccumulator native to the western United States, and is the most commonly used model hyperaccumulator in

the genus (Trelease and Trelease, 1938; Freeman *et al.*, 2006; Sors *et al.*, 2009). It is known only to inhabit seleniferous soils in the wild (Barneby, 1964). *Astragalus cicer* L. is an Old World species that has been introduced to the United States as a forage crop, in part due to its lack of seleniferous habit and broad tolerance of edaphic and climatic conditions (Acharya *et al.*, 2006). The “Oxley” ecotype used in this study that was first collected in the former USSR and introduced to the United States in 1971 (Acharya *et al.*, 2006). *Astragalus cicer* has been shown to accumulate little or no selenium in controlled greenhouse experiments (Davis, 1972), although in tissue culture its cells can be artificially selected to tolerate limited quantities of selenium (Wang *et al.*, 1999).

Greenhouse setup

We planted 98 seeds each of *A. cicer*, the non-accumulator (“Oxley” ecotype, propagated - Granite Seed, Denver, CO), *A. bisulcatus*, the hyperaccumulator (wild collected - Western Native Seed, Coaldale, CO), after physical scarification, on April 26, 2013 at Denver Botanic Gardens (DBG). After one month, we repotted plants, most of which had 1-2 true leaves, in 3.5 inch square pots in soil that was 3 parts Fafard® 4P Mix and one part Turface™. When plants were four months old, three plants of each species were randomly assigned to 12 treatments and arranged in a Latin Square Design. We dosed plants with sodium selenate solutions because selenate is readily bioavailable to hyperaccumulators (Shrift and Ulrich, 1969), and because it is the most common bioavailable form of selenium that hyperaccumulators might encounter in the field (Oldfield, 2002). Serial dilutions of sodium selenate in tap water were prepared (w/v)

such that each dilution contained 30-33% of the concentration of the previous dose, resulting in 12 different treatments from 100mg/L to 1µg/L sodium selenate, (i.e., 100mg/L, 30mg/L, 10mg/L, 3mg/L, 1mg/L, 300µg/L, 100µg/L, 30µg/L, 10µg/L, 3µg/L, 1µg/L, and tap water control). Sodium selenate is 41.8% elemental selenium by mass, such that the doses ranged from 41.8mg/L (529µM) to 0.418µg/L (5.29nM) of elemental selenium. Plants were watered exclusively with their treatment solution for the duration of the experiment, in order to resupply selenium lost by uptake or gradual chemical reduction to unavailable forms (Lu *et al.*, 2009). All plants were regularly watered generously to saturation and allowed to drain freely into hazardous waste containers, in order to elute any excess selenate buildup.

Tissue Concentration via ICP-MS

After drying and massing the plants, we removed approximately 25mg of dried young whole leaf tissue from each plant and pulverized it in a ball mill. We precisely massed between 1 and 10mg of powdered tissue from each sample and added 750µl concentrated nitric acid, 250µl concentrated hydrochloric acid, 100µl concentrated hydrofluoric acid, and 100µl concentrated hydrogen peroxide, all of which were trace metal grade (Thermo Fisher Scientific). Samples were then high-pressure digested in a Milestone Ethos EZ (Shelton, CT) microwave digester at 210°C for 21 minutes. Samples were then diluted to 15ml with > 18.0 MΩ cm water and analyzed via Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) with an Aglient 7700x (Santa Clara, CA). Microwave digested acids with no plant material and the National Institute of

Standards and Technology's Standard Reference Material 1570a "Trace Elements in Spinach Leaves" were used as negative and positive controls, respectively. The method detection limit for the ICP-MS analyses was $0.53 \mu\text{g Se L}^{-1}$, and samples ranged from $0.72 - 716 \mu\text{g Se L}^{-1}$.

Plant performance

Plant leaf number and stem length were measured weekly during the treatment period until the final measurement on November 8, 2013 when plants were 7 months old and, by our stem and leaf measurements, no longer appeared to be growing substantially. None had flowered. After the treatment period ended, plants were removed from soil, cleaned, and dried. Aboveground and belowground parts were separated and massed. To account for pre-treatment differences in size, we analyzed the net leaf proportion and net stem growth of plants by subtracting the initial value of leaf number and stem length, respectively, from the final values and then dividing by the initial values.

Herbivory

Spider mites are common greenhouse pests that are typically well controlled by overhead watering, but spread quickly in xeric plants or plants watered at the base. Due to our hand watering, we had a persistent spider mite (*Tetranychus urticae*) infestation during the treatment period. Because DBG is primarily a propagation greenhouse – not an experimental greenhouse – we were not permitted to let the infestation proceed unchecked. We attempted control by periodically inverting each plant and spraying the

shoots and leaves with water. The entire greenhouse was fumigated twice during the growing period with the biological insecticides BotaniGard® ES and Aza-Direct, for additional arthropod control.

To quantify degree of herbivory, on November 1, one week before the final harvest, we took digital images of the youngest fully expanded leaf on the main stem of each plant (representing approximately 5-20% of total leaf area, on average). Because spider mites are cell disruptor herbivores which cause yellowed spots wherever they have fed, we used ImageJ v1.48 and the color threshold tool to calculate the proportion of each leaf that was damaged by herbivory. Images were anonymized and randomized before analysis to eliminate experimenter bias. Images with ambiguous damage/senescence were excluded.

Statistical Analysis

All data sets were analyzed via JMP v11. The two species were analyzed together with species as a model effect. To investigate how species and soil Se dosage predict tissue Se concentration and how species and tissue Se concentration predict herbivory, we used ANCOVA with Se dosage and tissue Se as the covariate, respectively. For both of these tests, $\log_{10} + 0.0001$ transformations were applied to both independent and dependent variables to improve normality, since these data sets were highly right-skewed, contained zeros, and spanned several orders of magnitude. We chose this transformation rather than the more standard $\log_{10} + 1$ transformation because our datasets contain values both greater than and less than 1, and a $\log_{10} + 1$ transformation artificially

compresses values less than 1 relative to values greater than 1, resulting in a dataset that remains right-skewed. To investigate the ability of the three factors, species, herbivory, and soil dosage, to predict plant performance metrics, we ran two separate ANCOVAs, since herbivory and soil dosage are highly correlated. In both instances, species was the categorical model effect and either herbivory or soil dosage was analyzed as the covariate. For these two analyses we left both independent and dependent variables untransformed, since the dependant variables were approximately normal. We also tested the effects of tissue Se and bioconcentration factor (the ratio of soil dosage to leaf concentration) on these same plant performance metrics. We ran two additional ANCOVAs using species as the categorical model effect and either $\log_{10} + 0.0001$ transformed tissue Se or $\log_{10} + 0.0001$ transformed bioconcentration factor as the covariate. Because of our uncertainty about background levels of selenium in our materials, and thus the accuracy of the bioconcentration factors for the lowest dosages, we excluded plants dosed with less than 0.1mg/kg Se for the bioconcentration factor analysis.

Results

Soil dosage versus tissue concentration

Both *A. cicer* and *A. bisulcatus* accumulated substantial amounts of selenium. *A. cicer*, the non-accumulator, accumulated as much as 1052 mg/kg of selenium in its dry mass. *A. bisulcatus*, the hyperaccumulator, generally accumulated at least an order of magnitude more than *A. cicer*, ending the treatment period with Se representing as much as 10,000mg/kg or more of total dry mass. There was a high degree of individual variability within treatments, with as much as 5-fold differences between *A. cicer* individuals and 10-fold differences between *A. bisulcatus* individuals at the same dose (Figure 3.1, Figure S3.1). 3% of *A. cicer* individuals surpassed the 1000mg/kg threshold, 30% surpassed only the 100mg/kg threshold, while 67% surpassed neither. For *A. bisulcatus*, 25% surpassed the 1000mg/kg threshold, 19% surpassed only the 100mg/kg threshold, and fully 56% failed to surpass either – a distribution that is strikingly similar to that of wild-collected *A. bisulcatus* plants at ~20%, ~30% and ~50%, respectively (Shrift, 1969).

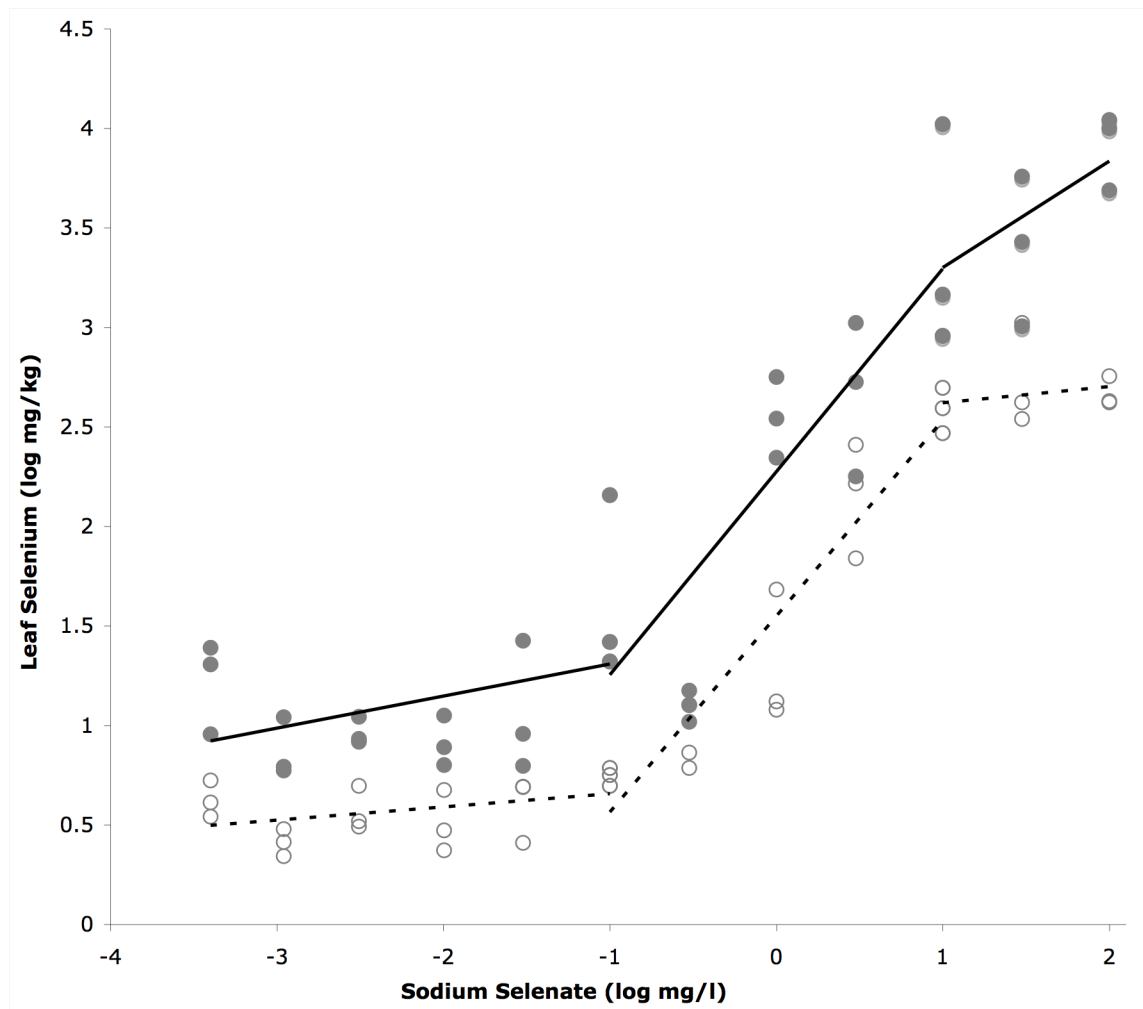


Figure 3.1: Accumulation curves of log-transformed whole leaf selenium concentration versus log-transformed soil dosage of sodium selenate. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Linear regression analyses were broken into three segments, 0-0.1mg/l, 0.1-10mg/l, and 10-100mg/l. Solid lines represent best fit lines for *A. bisulcatus* and dashed lines represent best fit lines for *A. cicer*. Species was a significant factor in all three segments, but dosage was only significant for the 0.1-10mg/l segment.

By ANCOVAs, both *A. cicer* and *A. bisulcatus* had leaf concentrations of selenium that, although different from one another with means of 4.0mg/kg and 20.2mg/kg, respectively, each remained flat when dosed with between 0 and 100µg/L of sodium selenate (species $P < 0.001$, log dosage $P = 0.11$, interaction $P = 0.49$, $n = 36$, $R^2 = 0.52$) (Figure 3.1). Between 100µg/L and 10mg/L sodium selenate, however, the

concentration of selenium in both species rose rapidly, up to an average of 394.6mg/kg for *A. cicer* and 4287.3mg/kg for *A. bisulcatus* (species $P<0.001$, log dosage $P<0.001$, interaction $P=0.86$, $n=30$, $R^2=0.80$) (Figure 3.1). Notably, in this range, which represents the vast majority of seleniferous native soils (Oldfield, 2002), the leaf selenium content of the two species was not distinguishable by simple T-test ($P=0.18$, $n=30$, $df=1$). However, bioconcentration factor, i.e. the ratio of leaf selenium to bioavailable soil selenium, was significantly greater for *A. bisulcatus* (805:1) than for *A. cicer* (97:1) ($P<0.01$, $n=30$, $df=1$). From 10mg/L to 100mg/L, the accumulation of selenium in both species flattened out once more at an average leaf concentration of 490.7mg/kg in *A. cicer* and 5356.4mg/kg in *A. bisulcatus* (species $P<0.001$, log dosage $P=0.10$, interaction $P=0.21$, $n=24$, $R^2=0.71$) (Figure 3.1).

Tissue concentration versus herbivory

There was a significant interaction between species and tissue concentration of selenium in predicting herbivory (ANCOVA, species $P<0.001$, log tissue Se $P<0.001$, interaction $P<0.001$, $n=59$, $R^2=0.60$). In other words, although both species experienced declining herbivory with increasing tissue concentrations of selenium, *A. bisulcatus*, the hyperaccumulator, was relatively well protected from herbivory across all leaf concentrations, while *A. cicer*, the non-accumulator, was poorly protected from herbivory at low leaf concentrations but well protected at the higher concentrations (Figure 3.2).

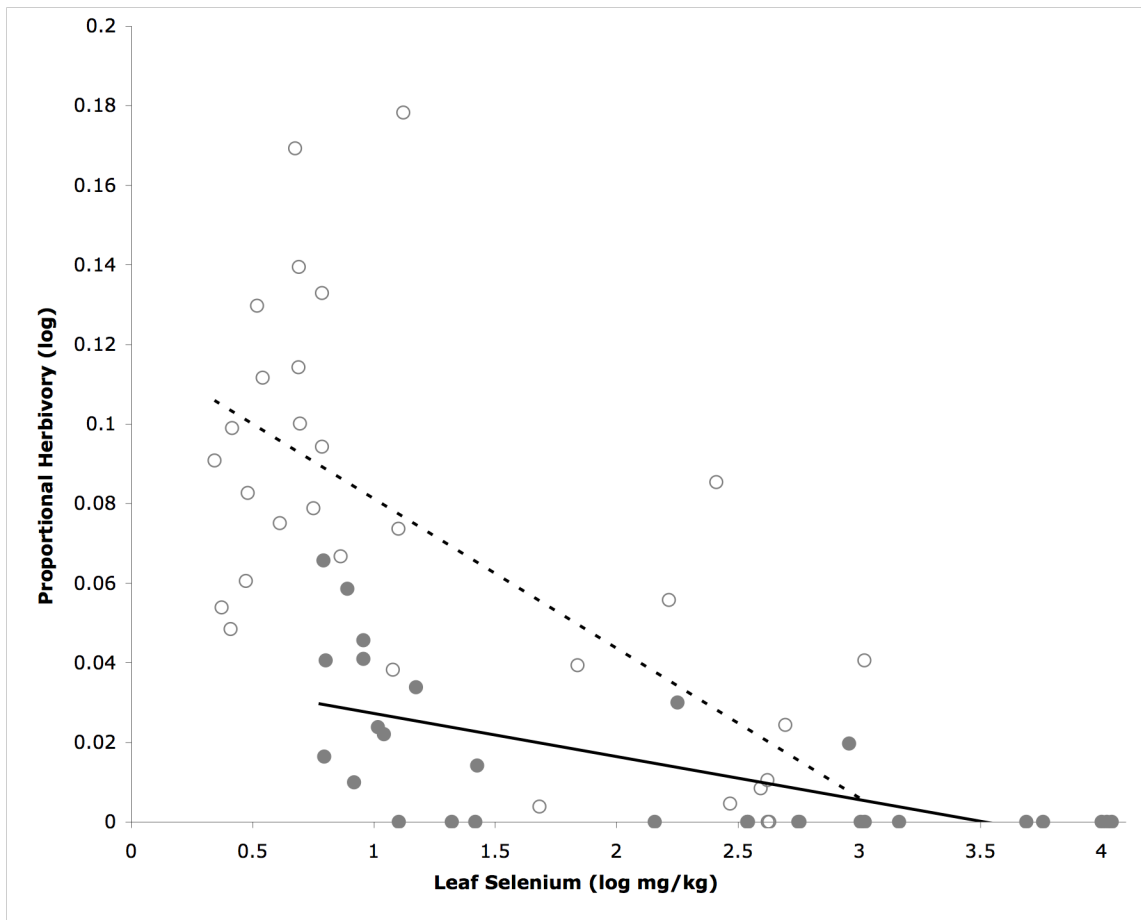


Figure 3.2: Plot of log-transformed herbivory (proportion of leaf area damaged) versus total log-transformed leaf selenium concentration. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Solid lines represent best fit lines for *A. bisulcatus* and dashed lines represent best fit lines for *A. cicer*. There was a significant interaction between species and leaf concentration in predicting herbivory, although both species had significant declines in herbivory with increasing tissue selenium.

Plant performance

Because of a strong colinearity between selenium variables and herbivory, we ran separate analyses to evaluate the ability of each variable to predict plant performance metrics.

Herbivory versus plant performance

There were no significant effects of herbivory or interactions between herbivory and species on any plant performance metric (ANCOVA, Table 3.1), although some metrics appeared to be trending toward significance.

Table 3.1: ANCOVA table of species and dosage on plant performance metrics. Non-significant p-values are grayed.

	Species	Dosage	Species*Dosage	n	R ² adj.
Aboveground Mass	P<0.001	P<0.001	P=0.48	72	0.58
Belowground Mass	P<0.001	P=0.90	P<0.05	72	0.49
Root/Shoot Ratio	P<0.001	P<0.05	P<0.01	72	0.17
Net Leaf Proportion	P<0.001	P<0.001	P<0.001	72	0.60
Net Stem Growth	P=0.05	P=0.07	P<0.05	72	0.11

Soil dosage versus plant performance

There were significant effects of selenium dosage and/or dosage by species interactions for all plant performance metrics investigated (ANCOVA, Table 3.2). Although *A. cicer*, the non-accumulator, had greater aboveground biomass across all treatment groups than *A. bisulcatus*, the hyperaccumulator, both species grew larger with increasing selenium dosage (Figure 3.3a). However, there was an interaction between species and dosage in the effect on belowground biomass, with *A. cicer* having no change in root biomass but *A. bisulcatus* having increased root biomass at higher concentrations of soil selenium (Figure 3.3b). Consequently, there was also an interaction between species and dosage in predicting root/shoot ratio. *A. cicer* showed a significant decline in root/shoot ratio with increasing selenium while *A. bisulcatus* showed no change (Figure 3.3c).

Table 3.2: ANCOVA table of species and herbivory on plant performance metrics. Non-significant p-values are grayed.

	Species	Herbivory	Species* Herbivory	n	R ² adj.
Aboveground Mass	P<0.001	P=0.07	P=0.86	60	0.51
Belowground Mass	P<0.001	P=0.98	P=0.28	60	0.43
Root/Shoot Ratio	P=0.10	P=0.16	P=0.17	60	0.36
Net Leaf Proportion	P=0.44	P=0.10	P=0.08	60	0.15
Net Stem Growth	P=0.99	P=0.08	P=0.37	60	0.07

Similarly, there were interaction effects for both net leaf proportion and net stem growth. While nearly all plants had a net loss of leaves over the treatment period, *A. cicer* plants at higher doses of selenium lost more leaves than those at lower doses, while the opposite was true for *A. bisulcatus* (Figure 3.3d). *A. bisulcatus* plants at higher doses of selenium had more stem growth than those at lower doses, but there was no difference across *A. cicer* plants.

Most notably, no *A. cicer* plants at any concentration died during the treatment period. None of the three *A. cicer* plants at the highest concentration of sodium selenate (100mg/L) showed evidence of herbivory, but all displayed apparent stress, as evidenced by a reddish leaflet margin and rachis. This was not necessarily associated with a high leaf selenium concentration, as some plants at lower soil concentrations had similar or higher leaf concentrations but no red margin.

Thus, the substrate generalist *A. cicer* showed positive, neutral, and negative responses to selenium, depending on the performance metric, while the hyperaccumulator *A. bisulcatus* grew better with increasing selenium by every metric.

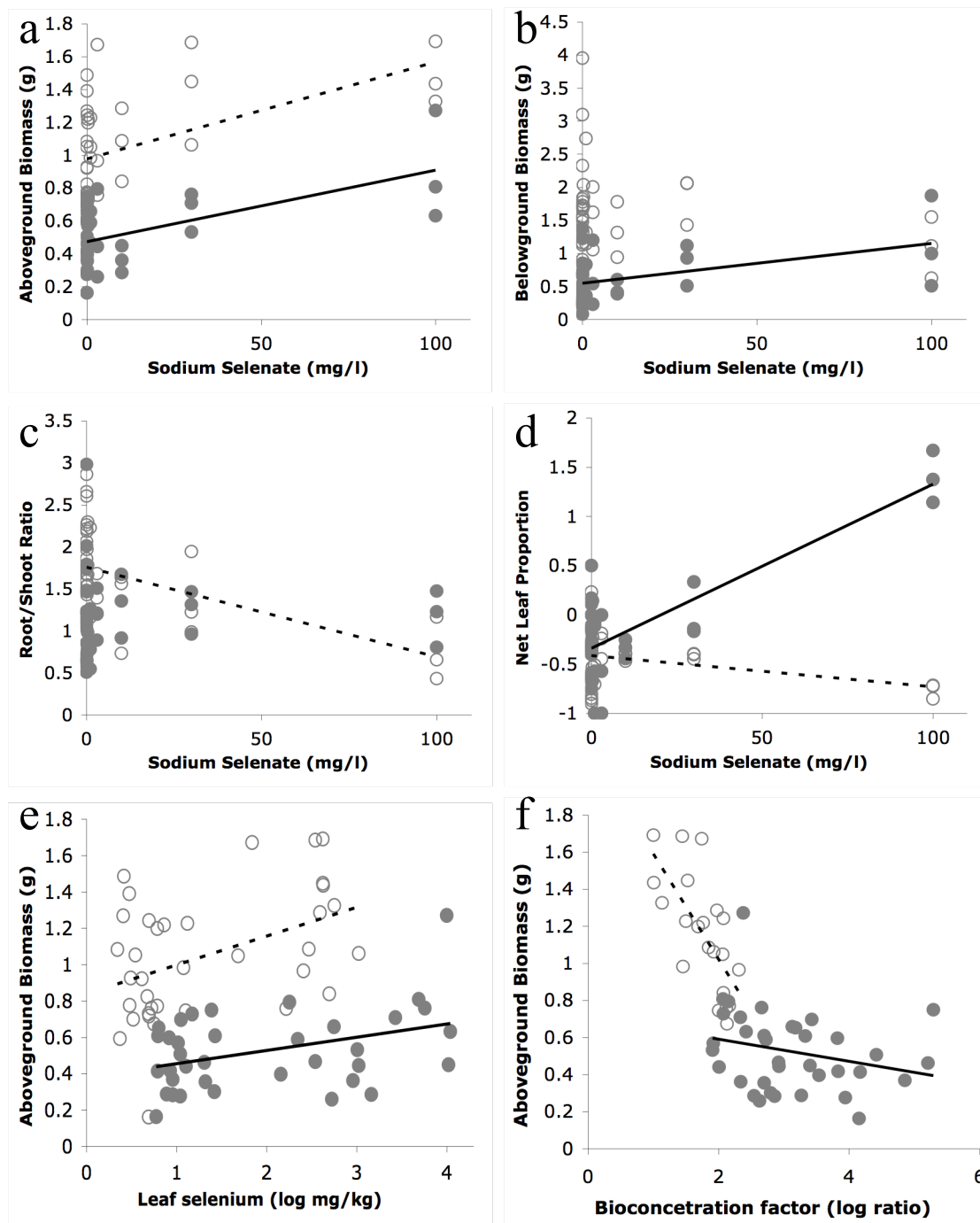


Figure 3.3: Plant performance plots for *A. cicer* and *A. bisulcatus*. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Untransformed sodium selenate dosage is plotted against the untransformed variables (a) aboveground biomass, (b) belowground biomass, (c) root/shoot ratio, and (d) net leaf proportion. (e) Log-transformed whole leaf selenium and (f) log-transformed bioconcentration factor are both plotted against untransformed aboveground biomass. Trendlines are included only for significant relationships.

Leaf selenium and bioconcentration factor versus plant performance

The effects of log-transformed leaf selenium concentrations on plant performance metrics were qualitatively and quantitatively similar to those of soil dosage (ANCOVA, Table S3.1, Figure 3.3e). Log-transformed bioconcentration factor, on the other hand, had nearly the opposite effect on plant performance (ANCOVA, Table 3.3). It was not a significant predictor leaf proportion, stem length, or belowground biomass, but aboveground biomass declined sharply and significantly for both species with increasing bioconcentration factor (Figure 3.3f). In fact, bioconcentration factor was a stronger predictor (by adjusted R^2) of aboveground biomass than either soil or leaf concentrations of selenium. The interaction between bioconcentration factor and species was significant, meaning that *A. cicer*, the non-accumulator, is more negatively impacted by increasing bioconcentration factor than is *A. bisulcatus*, the hyperaccumulator. Driven by the loss of aboveground biomass, the root/shoot ratio increased significantly for both species with increasing bioconcentration factor, and the interaction effect again indicates a stronger negative impact on *A. cicer*.

Table 3.3: ANCOVA table of species and log-transformed bioconcentration factor on plant performance metrics. Non-significant p-values are grayed.

	Species	Bioconcentration	Species* Bioconcentration	n	R ² adj.
Aboveground Mass	P<0.01	P<0.001	P<0.05	42	0.68
Belowground Mass	P<0.001	P=0.72	P=0.21	42	0.52
Root/Shoot Ratio	P<0.01	P<0.05	P<0.01	42	0.29
Net Leaf Proportion	P=0.10	P=0.93	P=0.08	42	0.11
Net Stem Growth	P<0.05	P=0.46	P=0.73	42	0.08

It should be noted that there is substantial overlap between species in leaf tissue Se concentrations (Figure 3.3e), while there is very little overlap between species in bioconcentration factor (Figure 3.3f).

Discussion

Tissue concentration

We were surprised to find that the accumulation curves of both non-accumulators and hyperaccumulators differed from the predictions of the conceptual model (van der Ent *et al.*, 2013). Both plant species had a logistic pattern of accumulation, with no change in tissue concentration across the lowest dosages, a rapid rise at intermediate dosages, and plateauing concentrations at high dosages. This differed from both the logarithmic accumulation expected for hyperaccumulators and the flat exclusion of selenium expected for non-accumulators. The flat uptake at low levels of added Se may have several explanations. Our lowest treatment levels of Se may not have exceeded the soil's binding capacity, such that the Se was not biologically available until after the point when the soil became saturated. Alternatively, the background levels of selenium in our materials may have masked any changes in uptake at these low treatment levels. No solid potting media or municipal tap water can be entirely devoid of selenium, and hyperaccumulator seeds alone can contain more than 2000mg/kg selenium (Trelease and Trelease, 1938) (unless perhaps grown for several generations in ultrapure hydroponic solutions, as done for nickel by Brown *et al.*, 1987). As such, it is possible that both species theoretically do have a logarithmic accumulation curve that we did not see for

methodological reasons. It is also possible that differences in Se concentration at this small a scale ($<100\mu\text{g/L}$) are simply not biologically relevant. Still, we feel that the rapid rise and subsequent plateau for the hyperaccumulator *A. bisulcatus* are largely compatible with the model predictions.

In our non-accumulator, *A. cicer*, however, our results deviated dramatically from predictions. *A. cicer* was expected to maintain a consistently low concentration of selenium in its tissues across all soil dosages by actively preventing the uptake and transport of selenium until some threshold at which acute toxicity stunted or killed the plant (Rascio and Navari-Izzo, 2011). Instead, no *A. cicer* plants died, and the accumulation curve for *A. cicer* had the shape expected for a hyperaccumulator, albeit at a lower magnitude. One *A. cicer* individual even exceeded the 1000 mg/kg level typically cited as the hyperaccumulator threshold for selenium (Boyd, 2007), and eleven individuals exceeded the more lenient 100 mg/kg threshold (van der Ent *et al.*, 2013), although we grant that such thresholds are only considered valid for plants growing on native soils (Rascio and Navari-Izzo, 2011).

Still, we feel strongly that *A. cicer* is indeed a true non-accumulator - not a cryptic hyperaccumulator. True field hyperaccumulation in *Astragalus* is limited to two closely related clades containing 25 species (including *A. bisulcatus*) nested well within the monophyletic new world group known as Neo-Astragalus (Barneby, 1964; Wojciechowski, 2005). *A. cicer*, as an Old World species, is well separated from the hyperaccumulators by several hundred non-accumulating sister species and at least 4.4 ± 0.8 million years of divergence (Wojciechowski *et al.*, 1999, Wojciechowski, 2005).

Meanwhile, a broad taxonomic range of *Astragalus* non-accumulators have been shown to accumulate more than 200mg/kg when dosed in the greenhouse with as little as 1.6mg/L (20 μ M) selenium as selenate (Sors *et al.*, 2005). Even the model plant *Arabidopsis thaliana* can accumulate as much as 1000mg/kg when dosed with just 4mg/L (50 μ M) selenate (Zhang *et al.*, 2007). Indeed, many, if not most, plants seem to behave this way (Pollard *et al.*, 2014). Clearly, the phenomenon of induced accumulation in metaliferous soil is not unique to *A. cicer*.

There are several possible reasons *A. cicer* and other non-accumulators do not actively exclude selenium in greenhouse studies as had been predicted. First, non-accumulators, particularly those that are naïve to metaliferous environments, have little adaptive incentive to evolve active metal exclusion mechanisms. We might expect that only non-accumulating metalophytes (plants that habitually live on metaliferous soils) such as *Silene vulgaris* (termed “excluders” in the model of van der Ent *et al.*, 2013) would adaptively benefit from such mechanisms. Second, perhaps hyperaccumulation ability may not be as bimodal as the admittedly arbitrary concentration thresholds make it appear. Some argue that hyperaccumulation, at least for certain taxa, may not be a physiologically distinct phenomenon, but rather just the right tail of a lognormal distribution of uptake (van der Ent *et al.*, 2013). Although much more in-depth sampling of a range of species would need to be done to confirm or refute that hypothesis (similar to the methodology of White *et al.*, 2007, with more congeneric comparisons), the similar curve shapes between species and unexpectedly high tolerance of *A. cicer* do seem to provide some preliminary support for the idea. Finally, it may be the case that non-

accumulators do indeed act as excluders in the field, but not in the greenhouse, for reasons that are not yet clear, but could include differences soil texture, soil chemistry, selenium speciation, or the rhizosphere. Either way, the model prediction for “normal” plants may need to be revised from flat exclusion to sigmoidal or logarithmic uptake if common garden experiments and wild plant censuses find similar results.

Herbivory and elemental defense

Since the elemental defense hypothesis is already very well supported (Boyd, 2007), and has even been demonstrated specifically for *A. bisulcatus* and spider mites (Quinn, 2010), we were not surprised to find spider mite herbivory on *A. bisulcatus* decrease with increasing tissue concentrations of selenium. It is worth noting, however, that the significant negative relationship between tissue selenium and herbivory in *A. cicer* shows that selenium as an elemental defense can be effective even in generalist non-accumulator plants growing in relatively typical soil concentrations. It has been well documented that metals deter most herbivores even at relatively low concentrations in artificial diets (Coleman *et al.*, 2005; Cheruiyot *et al.*, 2013), in accumulators and hyperaccumulators (Hanson *et al.*, 2003; Hanson *et al.*, 2004; Behmer *et al.*, 2005, Quinn, 2010), and in excluder metalophytes (Ernst *et al.*, 1990). However, since generalist plants seem to store different chemical forms of metals than metal-adapted plants (Sors *et al.*, 2005) it is apparent from our results that even low concentrations of the less volatile inorganic compounds characteristic of generalists can be an effective defense. Thus, our results support the “defensive enhancement hypothesis”, or the idea that the first

generalist plants capable of colonizing toxic substrates could have received an immediate, albeit small, defense against herbivory, even before the evolution of true hyperaccumulation or metalliferous habit (Boyd, 2012).

Perhaps the more unexpected finding is that for a given leaf concentration of selenium, *A. bisulcatus* has less herbivory than *A. cicer*, at least up until about 1000mg/kg, when the two converge near zero herbivory (Figure 3.2). This finding has at least two possible explanations: either the hyperaccumulators had additional defenses that the non-accumulators did not, or the hyperaccumulators stored Se in forms (e.g., methylselenosysteine, selenomethionine, selenosystathionine, etc.) that caused stronger deterrence than those in non-accumulators.

The first of these explanations seems unlikely, given that neither of the most common organic defenses found in *Astragalus* (aliphatic nitro and indolizidine alkaloids (Rios and Waterman, 1997)) have been found in *A. bisulcatus* (Williams and Barneby 1977). Although 263 (52%) of the North American *Astragalus* species tested contained detectable amounts of these compounds, only 1 of the 24 hyperaccumulator species tested contained them. This is not surprising, given inorganic/organic defense trade-offs: cheap, abundant elemental defenses might mean that hyperaccumulators can eschew more costly organic defenses (Boyd, 2007). However, this would put them at a disadvantage on selenium-poor soils, where they would be relatively undefended, and indeed, do not naturally occur. *A. cicer*, meanwhile, has been found to contain at least some toxic alkaloids (Rios and Waterman, 1997). It is still possible, however, that *A. bisulcatus* is

better defended due to leaf toughness, C/N ratio, moisture content, or some other factor that could create differential herbivory between species.

While these factors may contribute to defense in *A. bisulcatus*, we feel that the difference in herbivory is more likely related to selenium uptake. Selenium hyperaccumulators cause taste and odor aversion in mammals and insects, likely due to volatile organic selenium compounds, including dimethylselenide and others (Hanson *et al.*, 2003, Freeman *et al.*, 2007, Freeman *et al.*, 2009; Pfister *et al.*, 2010). Since *A. bisulcatus* and *A. cicer* differ drastically in their ratios of organic selenium metabolites to total selenium (Sors *et al.*, 2005), it is likely that for a given total tissue concentration of total selenium, the higher proportion of organic compounds in *A. bisulcatus* would cause stronger aversion, even though the higher proportion of inorganic selenium in *A. cicer* may actually be more toxic (Pickering *et al.*, 2003).

Plant performance

Contrary to our predictions and the findings of other studies (Trelease and Trelease, 1938; Broyer *et al.*; 1972a ; El Mehdawi *et al.*, 2012), we found that both *A. cicer* and *A. bisulcatus* responded positively to selenium dosage by at least one plant performance metric. We initially thought this was due to elemental defense, but herbivory did not play a statistically significant role in the performance of the plants, so other mechanisms are likely at play. While herbivory was a near-significant predictor of several plant performance metrics (Table 3.2), we believe this is more likely an artifact of the strong correlation between soil dosage and herbivory ($R^2=0.57$) than an indication

of type II error. Future 2-factor designs that vary dosage and herbivory independently should be performed to separate these effects.

For *A. cicer*, the seemingly contradictory responses to selenium (loss of leaves, no change in root mass or stem length, increase in aboveground mass) may be the result of a complex interplay between herbivory, toxicity, and other physiological responses. For *A. bisculatus*, since selenium dosage was a statistically significant predictor of all performance metrics and herbivory was not a significant predictor of any performance metric, we feel that elemental defense alone is insufficient to explain the adaptive benefit of hyperaccumulation. While elemental defense by selenium is clearly an important driving factor for hyperaccumulator growth and distribution in the field (Galeas *et al.*, 2008), we have demonstrated that it is likely not the only factor, and it may not even be the primary factor in instances when herbivory is not limiting fitness (Trumble and Sorensen, 2008).

Of the five evolutionary hypotheses for hyperaccumulation other than elemental defense, as summarized by Boyd and Martens (1998), three (tolerance by sequestration, disposal from the body via deciduous organs, and nonadaptive inadvertent uptake) do not predict improved growth with increasing selenium, and in fact, may predict the opposite. One hypothesis (interference, also called elemental allelopathy) only predicts improved growth when plants are grown in competition. The remaining hypothesis, drought resistance via increased osmotic potential, seems inadequate given that plants were regularly watered to saturation, and our preliminary data suggest that selenium provides no advantage to these species when drought stressed (Statwick, unpublished data). Thus,

only physiological benefit to the plant (Cappa and Pilon-Smits, 2014) appears sufficient to explain the improved growth with increasing selenium.

Still, although we found a positive relationship between soil or leaf selenium and biomass, we found a negative relationship between bioconcentration factor and biomass. This implies that despite the benefits to a plant of possessing selenium, there are costs to actively concentrating it in tissues, particularly for non-accumulators. Thus, depending on the environmental context, the physiological and ecological benefits of possessing selenium at high concentrations may or may not offset the metabolic costs of uptake, which perhaps helps explain some of the distribution and accumulation patterns of wild plants.

Concluding remarks

One must be cautious when applying the results of controlled studies to models of ecological dynamics in natural settings, since the advantage of being able to hold environmental variables constant is also a limitation. Although a greenhouse can effectively determine whether plants have the physiological capacity for certain behaviors, such findings are not necessarily ecologically relevant, especially if the conditions in the greenhouse are never encountered by the plant in the field. Keeping this caveat in mind, we suggest that the value of these data lies primarily in characterizing the physiological potential of hyperaccumulators and non-accumulators under idealized conditions.

Given the large range of leaf concentrations we measured in this experiment, both across and within treatments, and the substantial degree of overlap between species, we found that the tissue concentration threshold definition of hyperaccumulation was only marginal at delineating hyperaccumulators from non-accumulators. In this experiment, more than half of the hyperaccumulator individuals failed to meet even the most lenient tissue threshold for hyperaccumulation. Only 16 hyperaccumulator individuals exceeded 100ppm, yet 11 non-accumulator individuals also exceeded that same threshold. Of course, our greenhouse conditions may not accurately represent field conditions; ecological correlates may make the distinction between hyperaccumulators and non-accumulators more discreet in the wild. Still, given that previous studies of wild collected *A. bisulcatus* have found that as many as 40-60% of individuals have tissue concentrations less than 100mg/kg (Shrift, 1969; Sors *et al.*, 2009), we feel that a more thorough sampling of wild hyperaccumulators and their soils is warranted.

In this experiment, the bioconcentration factor (the ratio of tissue trace elements to bioavailable substrate trace elements), performed better than the absolute concentration threshold in delineating hyperaccumulators from non-accumulators. For example, during the period of rapid linear increase in leaf concentration for our hyperaccumulator and non-accumulator species (100µg/kg-10mg/kg sodium selenate, representing the bulk of “native soil” concentrations), the mean leaf concentration was not significantly different between our hyperaccumulator and our non-accumulator. The bioconcentration factors, on the other hand, remained similar across dosages and were significantly different

between species. *A. cicer* averaged a 97:1 ratio, while *A. bisulcatus* averaged an 805:1 ratio.

Some authors advocate that the bioconcentration factor, while not without some technical issues (see van der Ent *et al.*, 2013), may be a better indicator of hyperaccumulation ability than the absolute threshold (Hobbs and Streit, 1986; Zayed *et al.*, 1998). It may even be a more ecologically relevant standard than a tissue concentration threshold, since concentration thresholds only measure metal tolerance, not necessarily accumulation ability *per se*. A potential limitation of the threshold definition is that there are many metallophytes which tolerate extraordinarily high tissue concentrations of metals, but are certainly not hyperaccumulators (Hobbs and Streit, 1986; Ernst *et al.*, 1990; McGrath *et al.*, 2003; Rascio and Navari-Izzo, 2011; Pollard *et al.*, 2014; Goolsby and Mason, 2015). Instead, hyperaccumulators are physiologically united by their ability to actively (and apparently at some cost) take up metals through their roots and actively translocate those metals from their roots to their shoots (Rascio and Navari-Izzo, 2011, Cappa and Pilon-Smits, 2014). Both of these pathways, by definition, lead to an increased bioconcentration factor as compared to a non-accumulating plant – tolerant or otherwise. However, since our own study was greenhouse based and thus not necessarily representative of field conditions, we propose that a field-based comparison of these two definitions of hyperaccumulation should be conducted.

The Se/S ratio also has been proposed as a better discriminator between selenium hyperaccumulators and non-accumulators than the absolute tissue threshold. Due to

strong molecular similarity between selenium and sulfur and the enhanced ability of selenium hyperaccumulators to discriminate between the two, selenium hyperaccumulators should have a substantially elevated Se/S ratio as compared to non-accumulators (White *et al.*, 2007). However, we were unable to test this hypothesis due to inherent limitations of sulfur detection by ICP-MS. This definition may also be of limited applicability to hyperaccumulators of other elements, as it is unclear what elemental ratios would be analogous.

Finally, we suggest that Broyer *et al.* (1972a)'s skepticism of selenium as a growth promoting element, coupled with limited historical collaboration between researchers of seleniferous and serpentine systems (compare Brown and Shrift, 1982 vs Baker and Brooks, 1989 and sources therein), has led to little serious consideration of the idea that hyperaccumulated elements can provide a direct benefit to plant growth. We grant that it has not yet been satisfactorily demonstrated that trace elements benefits plants directly rather than indirectly, such as through chelation of other toxic elements, facilitation of mycorrhizae, etc. (but see Broyer *et al.*, 1972a; Lindblom *et al.*, 2013). However, our study and recent others like it suggest that selenium (El Mehdawi *et al.*, 2012), nickel (Küpper *et al.*, 2001; Ghasemi *et al.*, 2014; Pollard *et al.*, 2014; Kazemi-Dinan *et al.*, 2015), and perhaps even cadmium (Roosens *et al.*, 2003; Kazemi-Dinan *et al.*, 2015) can benefit hyperaccumulators of these elements across concentration ranges that span three or more orders of magnitude and reach well into the acutely toxic range for most organisms. We therefore suggest the new label “elemental stimulation” for this phenomenon, and believe it should be more thoroughly investigated.

**CHAPTER FOUR: TRACE ELEMENT HYPERACCUMULATION IS
BENEFICIAL BUT DOES NOT IMPROVE DROUGHT TOLERANCE IN
ASTRAGALUS SPECIES**

Summary

Background and Aims: Despite a lack of experimental support, enhanced drought tolerance is one of the commonly invoked hypotheses to explain trace element hyperaccumulation. Trace elements may have osmolytic and/or antioxidative properties that help hyperaccumulating plants reduce water loss or its resultant damage. Selenium in particular seems like a promising candidate because of its known antioxidative and drought protectant properties in non-accumulators, but it has not been tested in hyperaccumulators.

Methods: Here we investigate the drought tolerance hypothesis in a controlled greenhouse setting using a full factorial design with seedlings of a selenium hyperaccumulator in the genus *Astragalus* and a non-accumulating congener.

Results: While selenium generally aided plants and drought harmed them, we found no evidence that selenium improved the drought tolerance of either species.

Conclusions: Drought tolerance appears to not be a universal mechanism to explain the evolution of hyperaccumulation, although it may still operate in some as-yet untested circumstances.

Introduction

Hyperaccumulation is a phenomenon in which plants take up toxic trace elements from the soil and sequester high concentrations of those elements in their tissues. Although hyperaccumulation is energetically costly (Statwick *et al.*, 2016), our understanding of its adaptive significance is incomplete. Only one hypothesis has been studied in any detail: that hyperaccumulated elements protect plants from herbivores and pathogens, termed “elemental defense” (Boyd 2007; Trumble & Sorensen 2008; Cheruiyot *et al.* 2013; Hörger *et al.* 2013). The remaining hypotheses in the literature are relatively unexplored, although recent evidence supports both elemental interference/allelopathy – the idea that hyperaccumulators can reduce competition by locally toxifying the soil (El Mehdawi *et al.* 2011; El Mehdawi *et al.* 2012; El Mehdawi *et al.* 2015) – and “elemental stimulation” – the idea that hyperaccumulated elements can directly promote plant growth or reproduction (Kupper *et al.* 2001; El Mehdawi *et al.* 2012; Ghasemi *et al.* 2014; Pollard *et al.* 2014; Kazemi-Dinan *et al.* 2015; Statwick *et al.* 2016).

One of the more intriguing yet rarely tested adaptive hypotheses for hyperaccumulation is that it may improve drought stress tolerance (Cappa & Pilon-Smits 2014). Two of the most common soil types on which hyperaccumulators are found, serpentine and seleniferous, are generally xeric and/or prone to drought (Trelease 1942; Bhatia *et al.* 2005); hyperaccumulated elements could potentially act as osmolytes, increasing water uptake and/or decreasing water loss, or alternatively, they could stimulate upregulation of intrinsic drought resistance pathways (Cappa & Pilon-Smith

2014). Evidence in support of this hypothesis is mixed and incomplete at best. To our knowledge, only four studies have subjected hyperaccumulators to drought stress, and three were designed to test the effect of drought stress on uptake, rather than the effect of uptake on drought tolerance. In those three studies, hyperaccumulators were exposed to varying drought regimes, and their overall metal uptake was measured, with mixed results. One study found increasing uptake of nickel with increasing drought stress (Bhatia *et al.* 2005), a second found no change in uptake of nickel (Kachenko *et al.* 2011), and the third found both no change in uptake of cadmium and a decreasing uptake in zinc (Novo & Gonzalez 2013). Only Whiting *et al.* (2003) independently manipulated both drought status and metal availability, and they found no significant effect of nickel or zinc hyperaccumulation on drought tolerance.

Still, selenium hyperaccumulators remain untested, and there is reason to believe that they may act differently. Selenium at relatively low concentrations has been shown to benefit non-accumulating agricultural and forage crops, protecting them from drought, salt stress, senescence, and UV (Xue *et al.* 2001; Kuznetsov *et al.* 2005; Kong *et al.* 2005; Germ *et al.* 2007; Kostopoulou *et al.* 2009; Yao *et al.* 2012). The addition of selenium appears to increase drought tolerance through upregulated antioxidant pathways, including superoxide dismutase, tocopherols, proline, and glutathione peroxidase (Xue *et al.* 2001; Kong *et al.* 2005; Yao *et al.* 2012). However, Kuznetsov *et al.* (2005) found nearly the opposite – selenium treated plants were still protected from drought, but had less proline and peroxidase activity, not more. It is worth noting that the molecular mechanisms by which selenium acts in plants are not well understood.

Although glutathione peroxidase in animals is a selenoprotein, incorporating a selenocysteine at its active site, plants appear to have no selenoproteins at all (Fu *et al.* 2002; Novoselov *et al.* 2002; Lobanov *et al.* 2009). It is thus unclear if and how selenium directly interacts with peroxidases and other antioxidant pathways in plants.

It may even be the case that the benefits of selenium in response to stress are simply an example of chemical conditioning hormesis (*sensu* Calabrese *et al.* 2007). In this widely observed phenomenon, moderate exposure to a toxic stressor – any toxic stressor, not necessarily selenium – can be beneficial to an organism. The toxin prompts the organism to upregulate its bioprotective pathways, including antioxidative ones, which then protect that organism from other stressors such as drought (Mattson 2008). While hormesis means that moderate doses of toxin are beneficial compared to none, excessive doses are still acutely toxic; predictably, this is the pattern observed when drought-stressed non-accumulators are dosed with selenium (e.g. Kong *et al.* 2005; Yao *et al.* 2012). If the ability of selenium to protect plants from drought is simply due to its toxicity, rather than any unique properties of selenium *per se*, we would expect moderate selenium to benefit drought stressed non-accumulators as much if not more than hyperaccumulators, which are apparently not stressed by selenium, and thus would not necessarily experience hormesis.

Because the ability of selenium to protect hyperaccumulators from drought has thus far gone untested, we designed an experiment to examine this hypothesis, testing the interaction of drought and soil selenium for both a hyperaccumulator species and a congeneric non-accumulator species. We predicted that in all cases, plants would have

lower vigor and survivorship with increasing drought severity, and that, based on our previous work, selenium alone would have a positive impact on hyperaccumulators but a mixed impact on non-accumulators (Statwick *et al.*, 2016). If the drought tolerance hypothesis were correct, we would also predict that selenium-treated hyperaccumulators would be more resistant to drought than both non-selenium-treated hyperaccumulators and selenium-treated non-accumulators. If so, the evolution of hyperaccumulation may be driven by drought tolerance as well as herbivory defense.

Methods

Study Species

We used two species of *Astragalus* to investigate the drought tolerance of selenium hyperaccumulators. *Astragalus bisulcatus* (Hook) A. Gray is a relatively widespread hyperaccumulator native to seleniferous soils in the western United States, and is the most commonly used model hyperaccumulator in the genus (Trelease & Trelease 1938; Barneby 1964; Freeman *et al.* 2006; Sors *et al.* 2009). *Astragalus cicer* L. is an Old World species that has been introduced to the United States as a forage crop, in part due to its non-seleniferous nature and broad tolerance of edaphic and climatic conditions (Acharya *et al.* 2006). The “Oxley” ecotype used in this study that was first collected in the former USSR and introduced to the United States in 1971 (Acharya *et al.* 2006).

Greenhouse setup

Two hundred seeds of each species, *A. cicer*, the non-accumulator (“Oxley” ecotype, propagated - Granite Seed, Denver, CO) and *A. bisulcatus*, the hyperaccumulator (wild collected - Western Native Seed, Coaldale, CO), were physically scarified for optimum germination (Statwick, unpublished data) and planted in germination trays at the University of Denver’s greenhouse on January 24, 2014. On March 3, 2014, when plants had germinated and grown true leaves, plants were randomly assigned to treatments and repotted into SC10 Ray Leach Cone-tainers™ (Stuewe and Sons, Inc., Tangent, Oregon) containing 6 parts well-draining potting soil, 1 part perlite and 2 parts Turface™. After transplanting, seedlings were allowed to acclimate for two weeks before treatments were started on March 14, 2014.

Plants of each species were randomly assigned to one of six possible treatment groups based on the full factorial of two treatments: selenium (with or without) and drought severity (low, medium, and high). *Astragalus cicer* treatments had 20 replicates per treatment group, but *A. bisulcatus* treatments had between 13-15 individuals due to lower germination rates. Total sample size was 202 plants. Treatments were organized in a randomized block design and systematically rotated every week in order to minimize the effects of planting location. Every two weeks during the treatment period, measurements of stem length and leaf number were recorded, and it was noted if the plant appeared dead or dormant. Plants that appeared dead or dormant were still watered in accordance with their treatment, in case they were still alive. After the treatment period ended, on June 6, 2014, plants were individually harvested, dried, and massed.

Drought treatments

In order to create realistic drought treatments that mimic conditions a plant might experience in the wild, we obtained average March-June rainfall measurements from 30 years (1981-2010) of climate data using the PRISM Climate Group of Oregon State University model for three naturally occurring populations of *A. bisulcatus*: Gunnison Gulch, Colorado; Great Divide Basin Area, Wyoming; and Chouteau, Montana. We chose March through June because that was the period over which we conducted our study, but also because it is the period during which wild seedlings would be germinating and establishing. The average monthly rainfall was a relatively arid 3 cm, which translates to 85 ml of water per plant per month, given the size of our Cone-tainers. All three drought treatments were given the same overall volume of water each month, but higher severity treatments were given water less frequently: 21.25 ml of water four times per month (Low severity), 42.5 ml of water twice per month (Medium severity), and 85 ml water once per month (High severity). This approach simulates stress in arid environments better than varying total water received, and has been shown to be effective to create a gradient for plant response (Sher *et al.* 2004). In all cases, plants were watered incrementally to minimize drainage.

Selenium treatments

Although the threshold for selenium hyperaccumulation has been variously cited as either 100mg/kg or 1000mg/kg dry mass (Boyd 2007; Van der Ent *et al.* 2013), the

median tissue concentration for several species of field collected *Astragalus* hyperaccumulators, including *A. bisulcatus* is actually most likely somewhere between 150mg/kg and 650mg/kg (Shrift, 1969), and sometimes entire populations contain no individuals with tissue concentrations above 100mg/kg (Sors *et al.*, 2009). In our previous work (Statwick *et al.*, 2016), we observed that potted *A. bisulcatus* plants watered with a 3mg/L sodium selenate solution (15.9 μ M) had a mean tissue concentration of 588mg/kg, so that dosage likely approximates average seleniferous field conditions. At that same dosage, we observed that *A. cicer* individuals accumulated a mean tissue concentration of 164mg/kg, and showed no evidence of acute or chronic toxicity. We therefore exclusively used a 3mg/L sodium selenate solution (15.9 μ M) in tap water to irrigate our “with selenium” treatment, and tap water alone for our “without selenium” treatment. Since all drought treatments were given the same volume of water overall, all “with selenium” treatments received the same total mass of selenium over the treatment period.

Statistical analysis

Our primary interest was in whether the availability of selenium or the ability to take up selenium changed a plant’s response to drought, as detected by a significant interaction between selenium, drought, and/or species. If selenium uptake provides this benefit, we would expect those plants with selenium to be less negatively impacted by drought, and for the hyperaccumulator to receive a greater benefit than the non-accumulator, as measured by growth and/or mortality.

We used two different analyses to examine mortality in our plants. We compared the probabilistic survivorship curves between treatments by using a proportional hazards test, with a full factorial of selenium, drought, and species as factors. This analysis is intended for time series datasets composed of binary data in which each observation is an independent replicate (i.e. each plant has died or remains alive). It compares treatments via χ distribution, and calculates a risk ratio (RR) between treatments, where a RR greater than one means higher relative mortality and a RR less than one means lower relative mortality. Individuals that survived the entire treatment period were right-censored, while all other individuals were interval censored between the date they were last observed alive and first observed dead.

We also compared mean lifespan between treatments using a least squares linear model with a full factorial of selenium, drought, and species as factors. “Lifespan” was defined as the difference in days between the start of the treatment period and the date the plant was last observed alive. Plants that went dormant and subsequently regrew were counted as alive during dormancy. Plants that survived the treatment period were conservatively considered to have had a lifespan only equal to the length of treatment period, although it is probable that some would have lived considerably longer if permitted. Thus, for those treatments in which plants survived (all but the high drought severity treatments), mean lifespans are an underestimate.

We also analyzed six plant performance metrics using least squares linear models: maximum leaf count, change in leaf count, maximum stem length, change in stem length, aboveground biomass, and belowground biomass. For each performance metric, we used

a full factorial of selenium, drought, and species as factors. Since lifespan was a very strongly significant factor ($P < 0.0001$) in predicting all six plant performance metrics (i.e., plants that lived longer were larger on average), it was also included as a covariate in all six models.

All post-hoc analyses were done with Tukey HSD tests. All analyses were completed in JMP v.11.0.

Results

By the end of the study period, the vast majority of plants had died. The surviving nine *A. cicer* and eight *A. bisulcatus* individuals represented less than 10% of the original 202 plants. Individuals from both selenium treatments survived, but no individuals from the high drought severity treatment survived. However, some plants of either species went dormant for as much as 50 days during the treatment period before eventually re-growing leaves, so it is possible that there were some additional dormant survivors.

We found no significant interactions between the effects of species, drought severity, and selenium on any survivorship or plant performance metric, so the effects of each factor are described individually below. Nonetheless, the significance levels reported in Table 4.1 and for post-hoc tests are those from full-factorial models, rather than simplified single-factor models.

Study species

On average, *A. cicer* (the non-accumulator) lived 47% longer than *A. bisulcatus* (the hyperaccumulator) (Figure 4.1) and had higher survivorship during the study period (RR=0.70) (Table 4.1). In addition to having substantially higher germination rates (>60% vs. 41.5%), it also appeared more vigorous in general, with stems nearly twice as long on average and more leaves than *A. bisulcatus*. Despite the difference in stem length and leaf number, the slightly larger mean shoot biomass in *A. cicer* was not significant.

However, the differences in vigor between species were present pre-treatment, and *A. cicer* actually had proportionally less stem and leaf growth than *A. bisulcatus* over the treatment period. Additionally, at the end of the treatment period, *A. bisulcatus* individuals had an average of 41% more root mass than *A. cicer* individuals.

Table 4.1: Statistical table of experimental factors (columns) and response variables (rows). In all cases, the statistical tests were performed as a full factorial of all three experimental factors, but **no interaction terms were significant**, so only the effects of the individual factors from the full factorial test are reported below. Survivorship was analyzed with a proportional hazards test. Lifespan was analyzed via ANOVA. The remaining response variables were analyzed via ANOVA with lifespan as a covariate. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

Response variables:	Species			Drought			Selenium		
	F	n	DF	F	n	DF	F	n	DF
Survivorship	$\chi^2=5.35^*$	202	1	$\chi^2=6.88^*$	202	2	$\chi^2=2.57$	202	1
Lifespan	9.86**	202	1	3.22*	202	2	4.07*	202	1
Leaf Max	13.56***	175	1	1.01	175	2	0.19	175	1
Leaf Change	11.56***	170	1	0.16	170	2	0.03	170	1
Stem Max	78.01***	175	1	0.29	175	2	1.57	175	1
Stem Change	6.79**	170	1	0.34	170	2	1.55	170	1
Shoot Mass	3.49	158	1	1.28	158	2	4.27*	158	1
Root Mass	4.64*	130	1	3.82*	130	2	1.19	130	1

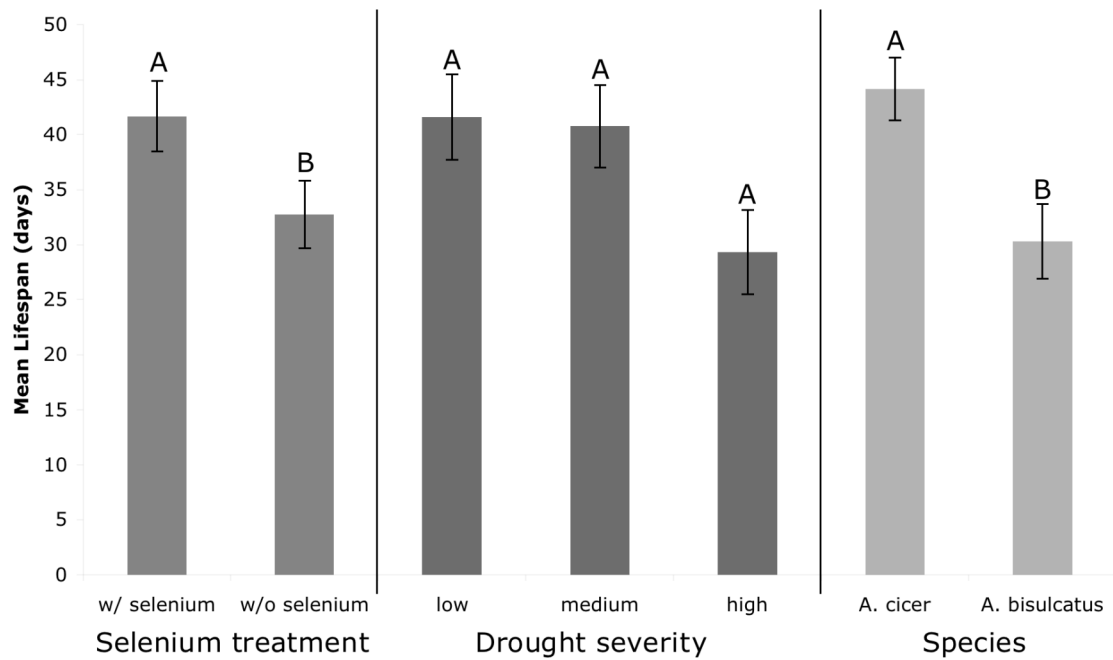


Figure 4.1: Least squares mean lifespan of plants grown in various treatments. This graph depicts the means within treatment groups of a full factorial least squares linear model of all three factors. **No interaction terms were significant**, so the treatments are depicted here separately. Letters denote significant differences between groups. Although drought severity was a significant factor in the overall model ($P < 0.05$), a *post-hoc* Tukey HSD test did not detect a significant difference between treatment groups.

Drought treatments

Drought severity was a significant predictor of survivorship during the treatment period (Table 4.1). Low drought severity plants had a significantly higher survivorship rate than either medium severity ($RR=0.70$) or high severity ($RR=0.62$) plants, although survivorship was similar for medium as compared to high severity ($RR=0.88$). Drought severity was also a significant factor in average lifespan, although a Tukey HSD test failed to find significant differences between treatments (Figure 4.1). However, the low severity treatment was nearly significantly different from both the medium ($P=0.085$) and high ($P=0.066$) severity treatments. Average lifespans in the medium and high severities

were nearly identical ($P=0.99$), although the medium severity treatments had five surviving individuals, while the high severity had none.

Drought severity was not a predictor of any aboveground characteristics, but it was a significant predictor of belowground mass. Mean root mass decreased with increasing drought severity, but only the low and high severity treatments were significantly different from one another by Tukey HSD ($P<0.05$, $n=81$, $DF=1$), while the medium severity was different from neither low ($P=0.12$, $n=88$, $DF=1$) nor high ($P=0.63$, $n=91$, $DF=1$) severity.

Selenium treatments

Plants exposed to selenium lived an average of 23% longer than untreated plants, regardless of drought treatment or species. Although selenium treated plants also had higher final survivorship (11 survivors w/ Se vs. 6 survivors w/o Se), the difference only approached significance by proportional hazards test ($RR=0.78$) (Table 4.1).

Selenium treated plants, despite their longevity, had 15% less aboveground mass on average than untreated plants (Table 4.1). There were no other significant effects of selenium on plant performance metrics.

Discussion

Contrary to the predictions of the drought tolerance hypothesis, we found no evidence that presence of selenium in the soil mitigates drought stress for either the selenium hyperaccumulator or the non-accumulator. This was true despite significant

effects of both drought and selenium treatments. Based on these results, it appears that drought tolerance may not be a substantial factor in the evolution and maintenance of the hyperaccumulation trait, at least for the seleniferous *Astragalus*. This agrees with Whiting *et al.* (2003)'s work in nickel and zinc hyperaccumulators, providing additional evidence against the drought hypothesis in general. While it is still possible that hyperaccumulation aids in drought tolerance for some species, some elements, or some environmental conditions, it appears as though the drought tolerance hypothesis is not satisfactory as a universal mechanism to explain the many independently derived evolutions of hyperaccumulation.

Our results do not preclude the possibility that selenium interacts with other environmental factors such as microbial communities, soil fertility, or plant community interactions to improve hyperaccumulator drought tolerance in the wild. Still, when controlling for these factors, selenium alone appears incapable of improving drought tolerance. It is also possible that our selenium concentrations were too low to detect a significant drought protection effect. However, since a) the mean 588mg/kg tissue concentration we measured in *A. bisulcatus* at this dosage in our previous work (Statwick *et al.*, 2016) closely mirrors the median tissue concentration of wild plants (Shrift, 1969), b) the median concentration of bioavailable selenium we have sampled in native seleniferous soils (total Se >2 mg/kg) is a surprisingly low 0.32 mg/kg (Statwick, unpublished data), and c) we measured a significant positive effect of selenium on lifespan in this work, we feel that our selenium concentration was both realistic and adequate. Additionally, if only plants at extraordinarily high soil or tissue concentrations

of selenium experience a drought tolerance benefit, then drought tolerance could conceivably adaptively reinforce hyperaccumulation after it had evolved, but would not be sufficient to explain the initial evolutionary transition from non-accumulator to hyperaccumulator.

It does appear, however, that the hyperaccumulator *A. bisulcatus* is a drought adapted species – more so than non-accumulator *A. cicer*. Despite being smaller and less vigorous than *A. cicer* in the well-watered pretreatment phase, *A. bisulcatus* had more stem and leaf growth during treatments, and ended the period with substantially greater belowground mass than *A. cicer*. Since even the low stress treatment represents an average year in an arid environment, this is consistent with our understanding of *A. cicer* as a relatively competitive habitat generalist and *A. bisulcatus* as a relatively stress tolerant habitat specialist.

We were not surprised to see a negative impact of drought severity on belowground biomass, lifespan, and survivorship, nor were we surprised to see a positive impact of selenium treatment on lifespan – for both species, due to hormesis or elemental stimulation or both. We were, however, surprised to see a slight but significant decrease in aboveground mass with added selenium, despite the increase in longevity. In our own previous work, we found a significant increase in aboveground mass with increasing selenium dosage for both *A. cicer* and *A. bisulcatus*, although that experiment tested substantially higher concentrations of selenium (Statwick *et al.* 2016). The present finding may be an example of a type I error, given the relatively small mass of the plants

(0.024g on average) and the error introduced by massing plants that had died several weeks prior (although lifespan was accounted for in the model).

If this finding is not spurious, however, it may have at least two possible explanations: 1) selenium reduces aboveground biomass for seedlings, but increases it for more established plants or 2) selenium increases aboveground biomass when plants are well watered, but decreases it when plants are under even mild drought stress. The first explanation seems unlikely, as Davis (1972) reported that *A. bisulcatus* seedlings in the absence of selenium were “weak and unthrifty” but became “thrifty and healthy” after selenium was added. The second explanation is diametrically opposite of the drought tolerance hypothesis, which would predict that the advantage of selenium uptake would be most pronounced under stress, not saturation. Either way, our previous research has shown that there appears to be a trade-off in how selenium accumulation affects aboveground biomass, whereby the benefit of possessing selenium may not always outweigh the metabolic cost of accumulating selenium (Statwick *et al.*, 2016).

Overall, we feel that selection for hyperaccumulation is complex and multifaceted, likely involving an interplay between metabolic costs, elemental defense, elemental allelopathy, elemental stimulation, and perhaps other unrecognized and/or untested phenomena. Nonetheless, given that selenium did not act as a drought protecting mechanism for the seedling stage - which has the highest mortality rate and is most susceptible to drought - we believe it is unlikely to do so in other life stages. These findings suggest that drought tolerance is unlikely to have been a selective factor for the development of hyperaccumulation, at least not for seleniferous *Astragalus*.

**CHAPTER FIVE: A REPORT TO CONCERNED PARTIES ON THE
TAXONOMY AND CONSERVATION OF *ASTRAGALUS RAFAELENSIS* AND
*ASTRAGALUS LINIFOLIUS***

Summary

Rare, narrowly endemic species are of conservation concern not only because of their small population sizes and limited habitat, but also because of an assumed lack of genetic diversity. We examined the microsatellite genetic diversity within and between two rare cryptic sister species of selenium hyperaccumulators, *Astragalus rafaensis* M.E. Jones and *Astragalus linifolius* Osterhout (Fabaceae), the latter of which is a questionable taxon. When geographic distance between populations is accounted for, the two species are not genetically distinct. We therefore propose that *Astragalus linifolius* be subsumed into *Astragalus rafaensis*, which has priority. Additionally, the morphological characteristics that have been proposed in the past to separate these species appear to fall along a continuum, rather than forming a discrete separation between regions or genetic clusters, and likely represent simple regional variation. This combined species has a substantially larger population size than either species did individually, but is still known from fewer than 30 reliably documented locations overall. We recommend that the populations in the San Rafael Swell of Utah be considered a unique management unit because of geographic isolation from the populations in

Colorado. Nonetheless, diversity within populations is relatively high and inbreeding is low, so conservation concern for this species should focus on minimizing current and future threats to its limited habitat.

Introduction

History and Taxonomy

Astragalus raphaelensis M. E. Jones (Fabaceae), or the San Rafael milkvetch, is a selenium hyperaccumulator first described in 1923 from a location in the eponymous San Rafael Swell in Utah (Jones, 1923). As a drought adapted species, its leaflets are usually absent, with most leaves being just a tough, naked rachis. The browned stems and leaves from previous years' growth form a prominent thatch at its base. Its typical papilionaceous flowers are white, tinged with pink-purple, and its woody pods are ~1.5cm long (Barneby, 1964) (Figure 5.1).

In 1928, five years after *A. raphaelensis* was described, *Astragalus linifolius* Osterhout, or the Grand Junction milkvetch, was described. Its type location is near Grand Junction, Colorado, about 150 kilometers due east of the type location of *A. raphaelensis*, which was the only location known at the time. *Astragalus linifolius* is nearly identical in appearance to *A. raphaelensis*, even to the trained eye, but *A. linifolius* was described as having white flowers, with just a purple tipped keel - lacking the pink-purple tinges of *A. raphaelensis* (Osterhout, 1928).



Figure 5.1: *Astragalus raphaelensis* growing in the San Rafael Swell in Utah, with close-up of flowers (inset). The plant is growing on a steep embankment of sparsely vegetated seleniferous soil. The flowers are primarily white, with pale pink tinges, particularly on the tip of the banner and the base of the wings. The keel tip is a darker pink-purple, as in *A. linifolius*. The tan stems at the base are growth from the previous year, and the grey stems hidden below those are from older years.

Both species are in the *pectinati* section of *Astragalus*, which is made up exclusively of selenium hyperaccumulators (Barneby, 1964). Both are thought to be very closely related to *A. toanus* M. E. Jones, which occurs west of *A. raphaelensis* and *A. linifolius* and ranges from northern Arizona through Nevada to eastern Oregon, including a few populations in northwest Utah (Barneby, 1964; Welsh, 2007; SEINet). *A. raphaelensis* and *A. linifolius* are also thought to be closely related, although somewhat less so, to *A. saurinus* Barneby, which occurs only in northeastern Utah, near Dinosaur

National Monument (Barneby, 1964; Welsh, 2007; SEINet). Since all four species are allopatric and morphologically “essentially alike”, it is possible that all four represent “phases of a single, discontinuously dispersed species” (Barneby, 1964). Still, *A. toamus* and *A. saurinus* are somewhat more morphologically distinct (Barneby, 1964), and not considered questionable taxa by NatureServe (2015), so we have not examined them herein, although it may be warranted to examine them in the future.

Table 5.1: Study Sites. We collected tissue from n individuals at each site. *A priori* species designations were based on Colorado and Utah Natural Heritage Program identification of the occurrences. The type locations for both species were described only in general terms, so the exact locations are unknown.

Site	Location	n	<i>a priori</i> Species	Notes and Alternative IDs
4210	Gunnison River	30	<i>A. linifolius</i>	
5893	Gunnison River	3	<i>A. linifolius</i>	
8267	Gunnison River	3	<i>A. linifolius</i>	Possible <i>A. linifolius</i> type location
8423	Gunnison River	26	<i>A. linifolius</i>	
9263	Gunnison River	30	<i>A. linifolius</i>	
14908	Gunnison River	30	<i>A. linifolius</i>	
4331	Dolores River	5	<i>A. raphaelensis</i>	Is possibly <i>A. linifolius</i> (CNHP, 2013)
6368	Dolores River	30	<i>A. raphaelensis</i>	
7630	Dolores River	30	<i>A. raphaelensis</i>	Is <i>A. linifolius</i> (Welsh, 2007)
8004	Dolores River	1	<i>A. raphaelensis</i>	Includes <i>A. linifolius</i> and hybrids (CNHP, 2013)
9127	Dolores River	21	<i>A. raphaelensis</i>	
3828	San Rafael Swell	35	<i>A. raphaelensis</i>	
28883	San Rafael Swell	30	<i>A. raphaelensis</i>	Possible <i>A. raphaelensis</i> type location

Few additional locations of either species (deemed “element occurrences” by NatureServe, herein “occurrences” for short) were discovered for nearly 50 years after the two species were described. From the late 1970s through mid-1980s, however, there was an explosion of newly discovered occurrences of *A. raphaelensis* and *A. linifolius*,

bringing the combined number of occurrences from three to almost 30 (SEINet), and essentially codifying the distributions as they are known today (Figure 5.2).

However, many of the individuals and occurrences, particularly along the Dolores River, Colorado, despite generally being called *A. raphaelensis*, have been alternately and contrarily identified as *A. linifolius* or even hybrids between the two (Welsh, 2007; Weber and Wittmann, 2012; CNHP, 2013) (Table 5.1).

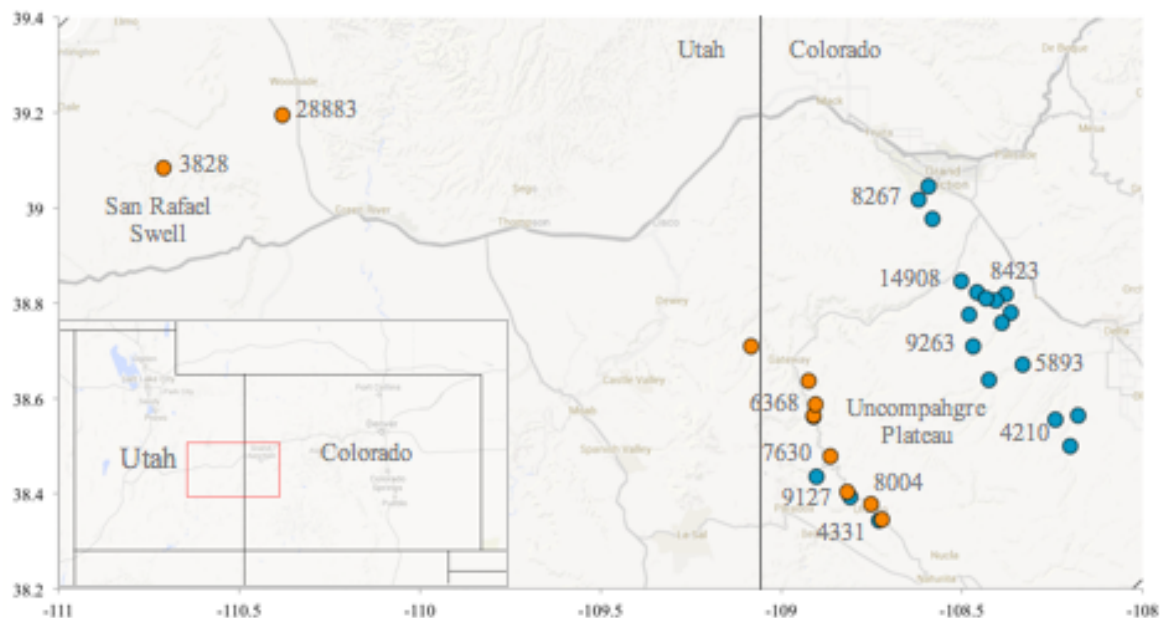


Figure 5.2: Known and sampled occurrences of *Astragalus linifolius* (blue) and *Astragalus raphaelensis* (orange), which are endemic to Western Colorado and Eastern Utah (inset). There are three main, disjunct regions in which the species grow, the San Rafael Swell in Utah (westernmost occurrences), the Dolores River Valley (central occurrences), and the Gunnison River Valley (easternmost occurrences). Plants west of the Uncompahgre Plateau, in the San Rafael Swell and the Dolores River Valley, have been typically regarded as *A. raphaelensis*, while those east of the Uncompahgre Plateau, along the Gunnison River Valley, are typically regarded as *A. linifolius*. However, plants along the Dolores River Valley are often morphologically ambiguous, and have been variously identified as both species and/or as hybrids, hence several blue sites in that area that largely overlap with orange sites. The locations in Colorado are based on CNHP tracked occurrences, and the locations in Utah are inferred occurrences based on UNHP point observations and SEINet herbarium specimens. Numbered occurrences are those that were sampled in this study.

Morphological traits other than flower color have been proposed to delineate between the two species, but taxonomic keys do not always agree on what these traits are and sometimes even disagree with the original species descriptions (Table 5.2). Commonly, *A. linifolius* is recognized as having erect pods, distinct from the reflexed pods on *A. raphaelensis*. Also, some authors indicate that the calyx teeth on *A. linifolius* are unequivocally longer than those of *A. raphaelensis*, yet other authors either omit calyx teeth, or indicate that the ranges of tooth sizes largely overlap (Table 5.2).

Astragalus linifolius was reduced to *A. raphaelensis* by Rydberg in 1929 on the basis of overall similarity. In contrast, C. L. Porter reduced *A. linifolius* (but not *A. raphaelensis*) to *A. toanus* in 1951 on the basis of its erect pods, even though *A. toanus* generally has purple flowers that are darker still than those of *A. raphaelensis* (Barneby, 1964). *A. linifolius* was then reinstated by Barneby in 1964, since it appeared to be “at least varietally distinct”, albeit with the caveat that “*A. linifolius* is so poorly known that reflections on its taxonomic status have little value”. When Welsh revised the North American species in the genus in 2007, he kept *A. linifolius*, arguing that more recent herbarium collections do not necessarily contradict the two-species arrangement.

Table 5.2: The morphological characteristics of *A. rafaensis* and *A. linifolius* according to the original species descriptions (Jones and Osterhout), systematic treatments of the genus (Barneby and Welsh), and well-regarded Colorado state floras (Weber & Wittmann and Ackerfield). *Osterhout neglected to mention pod orientation in his formal description of *A. linifolius* in 1928, but his field note on a specimen collected in 1921 (before either species had been described) stated that the plant seemed unique in that the pods were “not upright on the stem” as they are in *A. toanus*.

Authority	Key Characters	Species	Flowers	Pod Orientation	Calyx teeth
Jones (1923)	-	<i>A. rafaensis</i>	light-purple or white and tinged with pink, scattered	reflexed	2mm
Osterhout (1928)	-	<i>A. linifolius</i>	white, keel with a small purple tip	not upright*	2mm
Barneby (1964)	flower color, pod orientation, calyx teeth	<i>A. rafaensis</i>	pink-purple, the wing-tips paler or white	declined or deflexed	0.8mm
		<i>A. linifolius</i>	white, the keel with a small purple tip	erect	1.4-2.8mm
Welsh (2007)	flower color, pod orientation	<i>A. rafaensis</i>	pale pink-purple	deflexed	1.1-2.1mm
		<i>A. linifolius</i>	white, keel tip pink purple	erect	1.1-2.8mm
Weber and Wittmann (2012)	pod orientation	<i>A. rafaensis</i>	white or pale purple	declined or deflexed	-
		<i>A. linifolius</i>	white, with purple spot on keel	erect or ascending	-
Ackerfield (2015)	pod orientation, calyx teeth	<i>A. rafaensis</i>	Pink-purple with white wing tips or white with a purple-tipped keel	pendulous (at least some)	0.8-1mm
		<i>A. linifolius</i>	white with a purple-tipped keel	ascending-erect	1.5-2mm

Life history, reproduction, and genetics

Both *A. raphaelensis* and *A. linifolius* have similar ecology and life history strategies. Both are drought-adapted perennials that hyperaccumulate selenium, and both can be found alongside the common plants of the Colorado Plateau like piñon, juniper, rabbitbrush, sagebrush, and saltbush, as well as other selenium specialist plants like other *Astragalus* hyperaccumulators and *Stanleya pinnata* Pursh (Barneby, 1964; CNHP, 2013). Their hyperaccumulation habit likely protects them from herbivory and disease, as well as increasing their growth (Statwick *et al.*, 2016), although it probably does not improve their drought tolerance (Statwick, Unpublished Data). However, they are consequently confined to soils with already elevated selenium, either on or very near seleniferous strata (Beath *et al.*, 1939). While *Astragalus* hyperaccumulators seem to grow adequately, if less robustly, on non-seleniferous soils in the greenhouse, they do not appear to do so in the wild, perhaps for ecological reasons (Statwick *et al.*, 2016).

These species likely have naturally low levels of gene flow between populations. In the rare European species *Astragalus exscapus* L., 90% of seeds are dispersed less than 50cm from the parent plant (Becker, 2010). There is little reason to suspect that *A. raphaelensis* or *A. linifolius* would behave differently, as both have pods that dehisce while still attached to the plant (Barneby, 1964). The small, smooth seeds are also unlikely to be animal dispersed, through either epizoochory or endozoochory. Although seeds from the non-seleniferous *Astragalus cicer* L. can remain viable after passing through the digestive system of a cow, their germination rate decreases substantially, to less than 3% within the first year (Willms *et al.* 1995). In addition, the seeds of *A. raphaelensis* and *A.*

linifolius are unlikely to be ingested in the first place. Selenium compounds cause taste aversion in grazing livestock (Pfister *et al.*, 2010), and rightly so - just 16 grams of leaves of the very closely related hyperaccumulator *A. bisulcatus* is sufficient to kill a sheep within a few hours of ingestion (Trelease and Trelease, 1937). The fruits of *Astragalus* hyperaccumulators, in addition to being woody, contain even more selenium than the leaves (Freeman *et al.*, 2006). *Astragalus linifolius* seems to be pollinated mostly by a narrow suite of native bees with relatively small foraging ranges (Karron, 1987). The plants are self-compatible and mildly autogamous (Karron, 1989).

The low levels of seed and pollen dispersal should theoretically result in high levels of inbreeding, as well as high degrees of structuring within and across populations. Surprisingly, Karron *et al.* (1988) found that the three occurrences of *A. linifolius* he knew of at the time had more polymorphism among allozyme loci and a lower inbreeding coefficient (F_{IS}) than expected, especially given how rare the species was thought to be. However, given that inbreeding depression was apparent in experimentally selfed crosses, Karron hypothesized that the species maintains low F_{IS} values through strong selection against inbred individuals (Karron, 1989).

Conservation status

With 28 documented occurrences total in Utah and western Colorado, *A. raphaelensis* is ranked as G2/G3 (globally vulnerable to imperiled) by NatureServe (2015), although it was most recently reviewed in 2002, and at that time, the most recent documented visit to any of the occurrences in Utah had been in 1985. *Astragalus*

rafaelensis is a Tier 2 Plant of Greatest Conservation Need on the Colorado Parks and Wildlife State Wildlife Action Plan (2015). The Action Plan lists the primary threats to *A. rafaensis* as habitat shifting, habitat alteration, and increased drought, all largely due to climate change; *A. rafaensis* is ranked as Extremely Vulnerable (the highest possible designation) on the Climate Change Vulnerability Index (CPW, 2015). Additionally, the Action Plan lists the lack of information about the complete distribution and population status as a potential threat to *A. rafaensis*. According to the Colorado Natural Heritage (CNHP) Program Rare Plant Guide (1997+), *A. rafaensis* is “Moderately Conserved”, and moderately threatened by development and the maintenance of roads and bridges.

Similarly, NatureServe (2015) lists *A. linifolius* as a G3Q species (globally vulnerable, but questionable taxonomy), based on data last reviewed in 1998. There are 21 documented occurrences, all within approximately 800km² in western Colorado. CNHP (1997+) lists the potential threats to *A. linifolius* as grazing, invasive weeds, motorized vehicles, and insect larvae infestations.

Both species are Colorado Bureau of Land Management (BLM) sensitive species.

Study aims

Our aims in this study were twofold. Our first aim was to use genetic data to resolve the taxonomic confusion surrounding these species. To do so, we examined the patterns of genetic structure across the entire ranges of both species and evaluated whether they support the current taxonomic arrangement or suggest an alternative arrangement. We also examined the morphological traits typically used to identify these

species and whether they correlate with any genetically identified clusters. Our second aim was to quantify the genetic diversity within and among occurrences of both species, in order to inform both threat assessment and management decisions. Additionally, we comment on the potential conservation threats we observed in the field when collecting our specimens.

Methods

Field Collection

In Colorado, an occurrence was equivalent to an element occurrence as tracked by the Colorado Natural Heritage Program. In Utah, occurrences were inferred based on point observations from the Utah Natural Heritage Program and herbarium specimens from SEINet. We collected leaf and stem tissue from 13 occurrences of both *A. rafaensis* and *A. linifolius* (Table 5.1, Figure 5.2). When considering only non-historical occurrences (those occurrences which have been verified extant in the last 20 years), we visited almost three quarters and collected tissue at fully half of them. Between one and 35 individuals were collected per occurrence (Table 5.1). We avoided collecting tissue from a plant growing less than 1m from another one we had already sampled, unless there were fewer than 30 individuals present. In that case, we collected tissue from every available individual. We stored the tissue in individually sealed coin envelopes within plastic bags filled with silica gel.

All Colorado collections were made in June of 2013. Fruits were used as the identifying character for most plants, allowing for differentiation of *A. rafaensis* and *A.*

linifolius from similar congeners; the species are sympatric with at least four other *Astragalus* (and other legume species) that have a superficially similar growth habit. Utah collections were made in May of 2015, while most plants had flowers and some had fruit. Vouchers and photos were taken at each occurrence to ensure accurate identification, and are deposited at the Kathryn Kalmbach Herbarium (KHD: Denver Botanic Gardens).

DNA Extraction and Genotyping

We extracted DNA from approximately 50mg of dried tissue using a GenCatch™ Plant Genomic DNA purification kit (Epoch Life Sciences, Texas, USA). We chose to use microsatellites for our phylogenetic aims in addition to our population genetic aims because traditional phylogenetic markers (chloroplast, ITS, etc.) show low bootstrap support and insufficient resolution for all but the most basal of infrageneric clades in *Astragalus* (Wojciechowski, 2005, Scherson et al., 2008). We screened and optimized the 17 microsatellite loci described for *Astragalus holmgreniorum* Barneby by King et al. (2012). Nine of the 17 loci were chosen for genotyping based upon preliminary optimization. Six loci showed reliable amplification and sufficient polymorphism when visualized by capillary electrophoresis, and are included in the analyses presented here (Table S1).

We ran 10µl PCRs on Mastercycler proS thermal cyclers (Eppendorf, Hamburg, Germany) using conditions largely as established by King et al. (2012). However, we used universal primer complexed dyes (Applied Biosystems DS-33 dye set, Foster City, CA, USA) to visualize fragments, so we tagged the forward or reverse locus primer with

an additional 5' universal primer (Table S1). We reduced the concentration of the tagged primer to 0.025 μ l per reaction and added an additional 0.25 μ l of the universal primer complexed dye. This prompted us to reoptimize the annealing temperature, MgCl₂, and BSA concentrations of the reactions, maintaining a 10 μ l total reaction volume (Table S1). We used Taq and standard buffer from New England Biolabs (Ipswich, MA, USA) and primers supplied by IDT (Coralville, IA, USA). The amplified PCR products were multiplexed into panels of three loci and run on an Applied Biosystems 3730 Sequencer with HiDi formamide and Liz500 ladder by the DNALab at Arizona State University. Occurrences were arranged within plates such that each plate contained at least one occurrence from each putative species, to eliminate platewise pseudoreplication artifacts. Data were scored in duplicate using PeakStudio v2.2 (<http://fodorlab.uncc.edu/software/peakstudio>) and Geneious (Biomatters Limited, Auckland, NZ).

Genetic Analysis

We used a series of independent analyses to determine genetic structure across the occurrences. We created pairwise distance matrices of F_{ST} (the relative proportion of the total genetic variation explained by population structure), Nei's standard genetic distance D (an estimate of the absolute divergence between populations), and geographic distance (great circle distance in km) using GenAlEx (Peakall and Smouse, 2006). Using these matrices, we performed Principal Coordinates Analyses (PCoA) (a multivariate ordination method to view the similarity between occurrences) using the ape package

(<http://CRAN.R-project.org/project=ape>) in R (<http://www.R-project.org>). We visualized the PCoA data using the ggplot2 package (<http://CRAN.R-project.org/project=ggplot2>), with 95% confidence ellipses created with the ellipse package (<http://CRAN.R-project.org/project=ellipse>). We also analyzed the distance matrices with an average linkage, Unweighted Pair Group Method with Arithmetic Mean (UPGMA), cluster analysis (a method of grouping occurrences by similarity and dissimilarity) using the vegan package (<http://CRAN.R-project.org/project=vegan>) in R.

We performed Bayesian cluster analysis using STRUCTURE (Pritchard et al., 2000), which determines a) given K genetic groups, which individuals align with which groups, irrespective of occurrence and b) how much admixture there is within individuals. We ran this analysis with five independent runs at each K with iterations of 10,000 for burn in and 100,000 for run length, for Ks of 1 to 14. The optimum K value was determined in duplicate with STRUCTURE HARVESTER (Earl and vonHolt, 2012) and the CLUMPAK beta (Kopelman et al., In Press).

In order to statistically compare the groupings identified by PCoA, cluster analysis, and STRUCTURE, as well as the *a priori* species groupings, we used Analysis of Molecular Variance (AMOVA) in Arlequin (Excoffier and Lischer, 2010), using 1000 permutations per test. AMOVA is conceptually similar to ANOVA in that it can test whether there is a significant difference between two or more groups, but it compares its test statistic to a permuted statistical distribution rather than an idealized one.

To determine the relationship between geography and genetic distance and structuring, we examined the correlation between geographic distance and genetic

distance (i.e. the matrices we created in GenAlEx, above) using the Mantel test, which is a linear regression of pairwise distance matrices that compares the test statistic to a permuted statistical distribution. We also used the Partial Mantel test (which tests the relationship between two variables after correcting for a third) to examine whether the genetic clusters we identified explained patterns of genetic distance, after taking geographic distance into account. We did both analyses using the vegan package in R with 1000 permutations per test.

Finally, in order to understand the levels of diversity, inbreeding, and uniqueness within occurrences and genetic clusters, we calculated occurrence and genetic cluster summary statistics using GenAlEx.

Since four of our occurrences had five or fewer individuals each and are therefore unlikely to be statistically representative samples, we excluded them from most analyses, but included them in STRUCTURE, which considers individuals independently and does not use occurrence as a prior. Geographic location was not used as a factor in any analysis except mantel testing.

Morphological characters

In order to assess the amount of morphological variation between and within the species and the extent to which that variation mirrors the *a priori* species and genetic clusters, we measured the length of five random calyx teeth from each of 47 individual plants, collected from across the range of the species. We measured 22 *A. rafaensis* and

25 *A. linifolius* plants. The measurements were made on herbarium specimens collected between 1915 and 2015, including the holotypes for both *A. rafaensis* and *A. linifolius*.

Of the 15 specimens from the Dolores River valley, 11 specimens were identified by their collectors as *A. rafaensis*, while four others were identified as *A. linifolius*, either presumably or explicitly on the basis of pure white flower color. *A. linifolius* specimens from this region are sometimes considered to be “misidentified” *A. rafaensis* plants, but this gave us the opportunity to investigate whether flower color and calyx tooth length are correlated.

Ten specimens were housed at KHD and were measured using digital calipers, alongside the three voucher specimens we collected in Utah for this study. The remaining 37 specimens were housed at the Intermountain Herbarium at Utah State University (UTC, n=15), the University of Arizona Herbarium (ARIZ, n=1), and the Rocky Mountain Herbarium at the University of Wyoming (RM, n=21). High-resolution digital images of these 37 specimens were measured using the measure tool in ImageJ v1.49 (<https://imagej.nih.gov/ij/>). There was no difference between measurement methods either overall ($P=0.89$) or within species ($P=0.45$, $P=0.76$, respectively), so we analyzed the data from the two methods together.

We analyzed all calyx tooth data using mixed liner models in JMP v.11.0 using individual plant as a random effect.

Results

Genetic structure

In short, the conventional, *a priori* scheme (Figure 5.2) is not supported by our genetic analyses. Instead, the data still indicate two genetic clusters, but those clusters largely align by state.

Principal Coordinates Analyses of pairwise F_{ST} and Nei's D values (Table S5.2) both show that the 95% confidence intervals for *A. linifolius* fall almost entirely within the confidence intervals for *A. raphaelensis* (Figures 5.3A, C). Confidence intervals by state, on the other hand, do not overlap (Figures 5.3B, D). In other words, *A. linifolius* is not different from *A. raphaelensis* by this analysis, but Colorado is different from Utah. These same state-wise groupings were also identified by cluster analysis of F_{ST} and D values; *A. raphaelensis* occurrences in Colorado were more similar to *A. linifolius* occurrences than they were to *A. raphaelensis* occurrences in Utah (Figure 5.4). The very high cophenetic correlation coefficients of the cluster analyses ($F_{ST} c=0.92$, $D c=0.92$) indicate that the dendrograms are fidelitous models of the relationships between occurrences. It is worth noting that by both PCoAs and Cluster Analyses, Colorado's *A. raphaelensis* occurrences are often even more similar to *A. linifolius* occurrences than they are to each other.

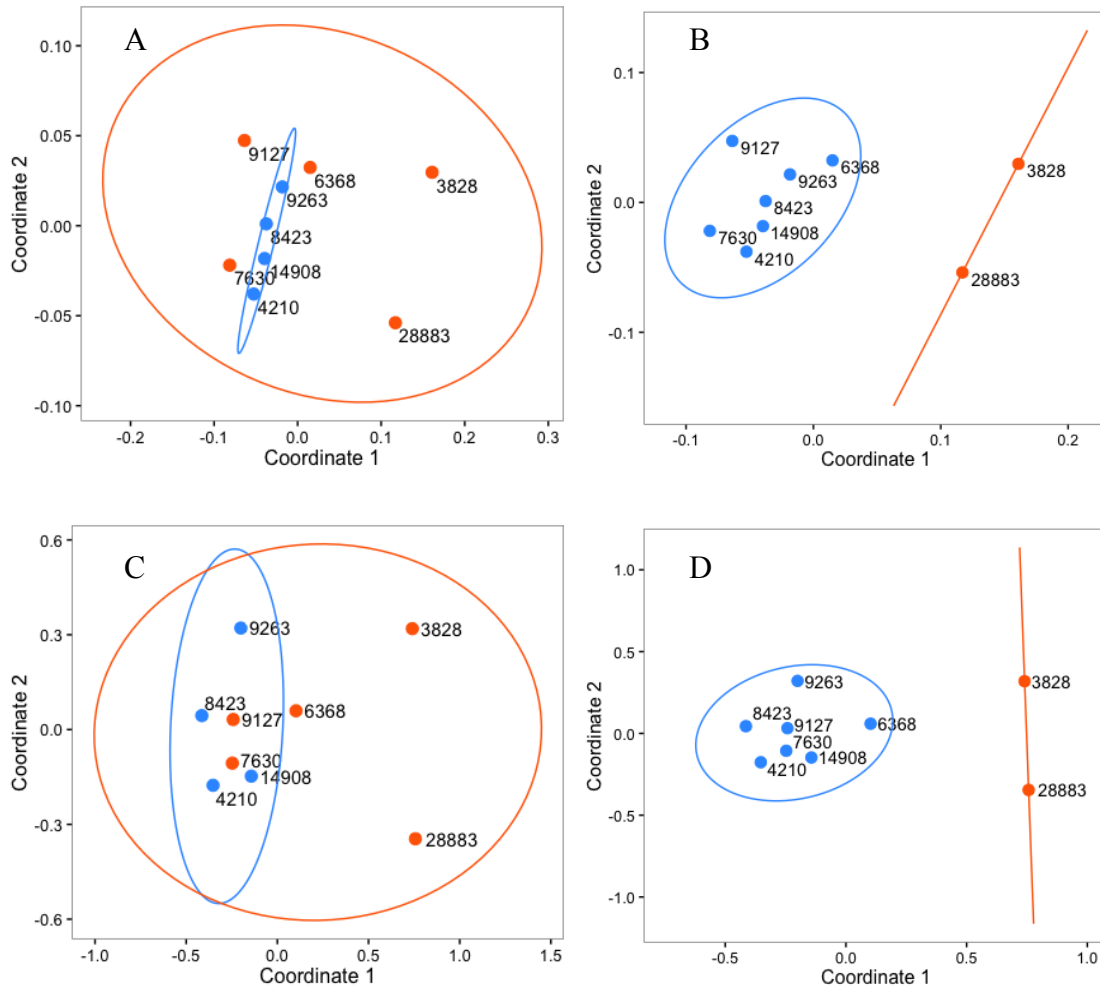


Figure 5.3: Principal Coordinates Analyses of F_{ST} (top, A and B) and Nei's D (bottom, C and D) values with 95% confidence ellipses. The left panels (A and C) show confidence ellipses for the *A priori* species clusters, with *Astragalus linifolius* in blue and *Astragalus rafaelsensis* in orange. The right panels (B and D) show confidence ellipses by state, with Colorado in blue and Utah in orange.

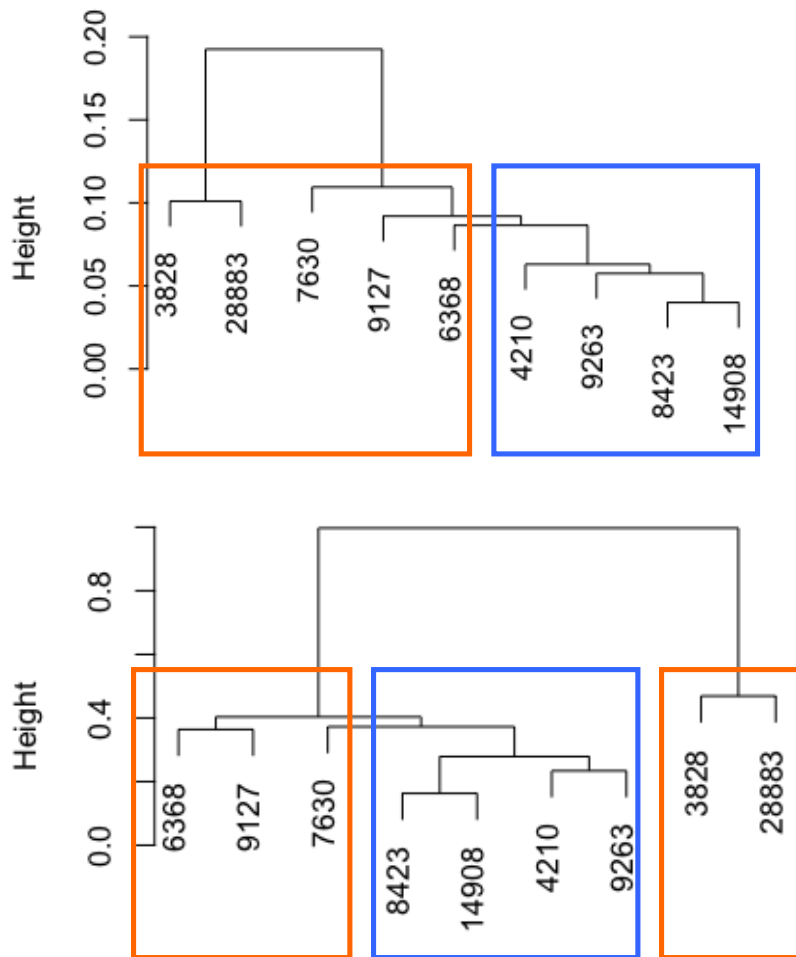


Figure 5.4: Average linkage cluster dendrograms of F_{ST} (top) and Nei's D (bottom) values. Occurrences that have historically been considered *A. linifolius* are highlighted in blue, while occurrences historically considered *A. raphaelensis* are highlighted in orange. Both dendrograms show that Colorado's *A. raphaelensis* occurrences (6368, 7630, and 9127) are more similar to *A. linifolius* occurrences than they are to Utah's *A. raphaelensis* occurrences (3828 and 28883).

The individuals we collected were grouped into two genetic clusters according to our STRUCTURE results; STRUCTURE HARVESTER and CLUMPAK each detected a probable K of two ($\Delta K=197.1$ for both). Instead of grouping the individuals by their *a priori* designations, the clusters group the individuals largely by state. There is considerable admixture between clusters, with 30.7% of all individuals showing a >10% mixed genetic signature, and 49% of Colorado individuals showing a >10% Utah

signature (Figure S5.1). Nine of the eleven Colorado occurrences show a >10% Utah signature, as do five of the seven occurrences with >20 individuals. The most western Colorado occurrence in particular, 6368, shows a majority Utah signal in STRUCTURE (Figure 5.5).

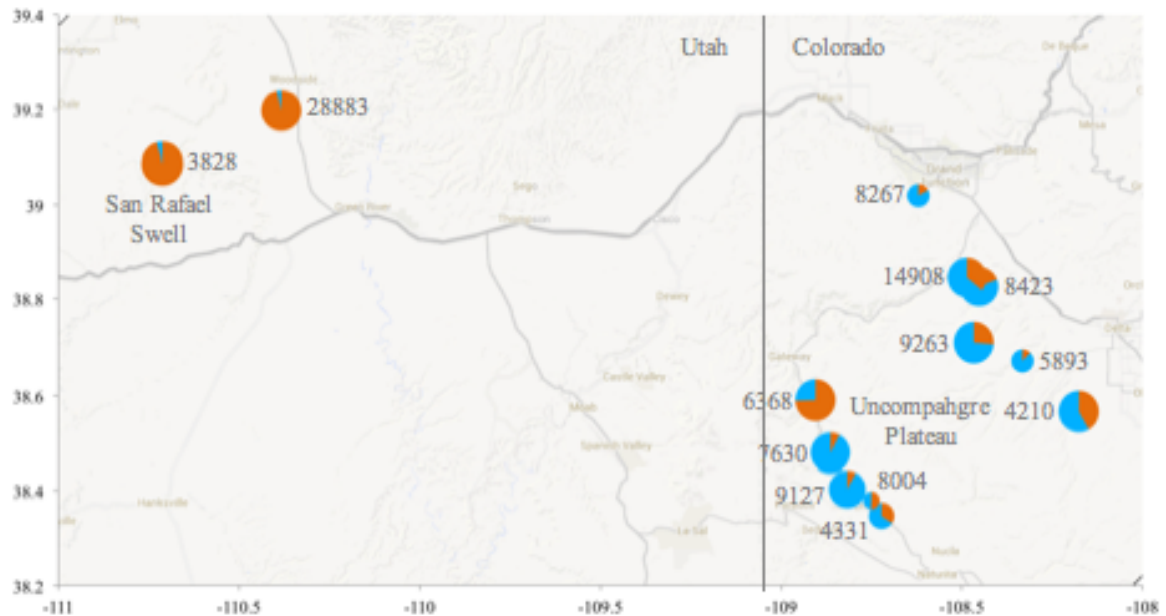


Figure 5.5: STRUCTURE groupings of sampled occurrences. Each pie chart represents one occurrence, with the blue portion representing proportional identity with Colorado genotypes and the orange portion representing proportional identity with Utah genotypes. The area of each circle is proportional to the square root of the number of individuals sampled at that site.

We used AMOVA to compare the different *a priori* and *post hoc* occurrence clusters identified by this study (Table 5.4). When we clustered the occurrences into the *a priori* conventional scheme of *A. linifolius* east of the Uncompahgre plateau and *A. raphaelensis* west of it (Figure 5.2) we find no significant difference between groups. We do find significant differences, however, when we compare either PCoA and cluster analysis groupings (Colorado vs. Utah) or STRUCTURE groupings (Colorado except 6368 vs. Utah plus 6368). By AMOVA, the state-wise groupings explain a higher

proportion of variation than do STRUCTURE groupings (Table 5.4), indicating that clustering occurrences by state is a better model for the genetic data.

Table 5.3: AMOVA table of different occurrence clustering schemes for the sampled occurrences. In the *a priori* species scheme, “*A. linifolius*” occurrences in the Gunnison River valley are compared to “*A. raphaelensis*” occurrences in the Dolores River valley and the San Rafael Swell (Figure 5.2). In the STRUCTURE groups scheme, occurrences were grouped into clusters based on their majority genotype assignment in the STRUCTURE results (Figure 5.5). In the Colorado vs. Utah scheme, occurrences in Colorado were compared to occurrences in Utah, as indicated by PCoA and cluster analysis (Figures 5.3 and 5.4). The F_{CT} value reported here is the proportion of the overall variation that is explained by the clusters, with a larger number indicating a better model of the data.

Test	d.f.	Sum of squares	F_{CT}	Significance
<i>a priori</i> species	1	28.513	0.019	P=0.26
STRUCTURE	1	55.918	0.087	P<0.05
Colorado vs. Utah	1	66.816	0.135	P<0.05

Evidence for two species?

None of our four independent tests of genetic structuring (PCoA, cluster analysis, STRUCTURE, and AMOVA) supported the *a priori* species clusters, and instead they supported genetic groups by state. Therefore, we investigated whether the state-wise genetic pattern we observed was still compatible with a two-species model (*A. raphaelensis* in Utah and *A. linifolius* in Colorado) or whether this pattern is better explained by one species with a disjunct distribution. We detected a very strong and significant signature of isolation-by-distance between occurrences for both pairwise F_{ST} and Nei’s D values ($r=0.88$, $P<0.01$; $r=0.91$ $P<0.01$; respectively) through Mantel testing. We found no significant difference between inter-state and intra-state comparisons by Partial Mantel testing for either F_{ST} or Nei’s D ($r=0.21$, $P=0.30$; $r=0.26$ $P=0.11$; respectively) when

geographic distance was the covariate, meaning that geographic distance alone is sufficient to explain the genetic difference between states.

Inbreeding and genetic diversity

All of the occurrences we surveyed had similar levels of inbreeding and genetic diversity (Table 5.5). The inbreeding coefficient was less than 0.15 for all but one of the occurrences, 8423, which had the highest genetic diversity by every other metric. The standard error of the inbreeding coefficient overlapped zero for six of the nine occurrences, indicating no statistically significant inbreeding. The three remaining occurrences whose standard errors did not overlap zero (8423, 9263, and 6368) were otherwise among the most diverse. Genetic diversity, in terms of average number of alleles per locus, effective number of alleles per locus, and Shannon Index, was generally highest in the northernmost occurrences in Colorado and lowest in the southernmost occurrences, with the two occurrences in Utah being roughly intermediate in diversity. Most occurrences had few private alleles (less than one per locus, on average), meaning little uniqueness at the individual occurrence level. The only two occurrences with more than one private allele per locus were 8423, the most diverse occurrence overall, and 6368, the Westernmost occurrence we tested in Colorado.

Because none of our clustering methods supported the *a priori* species, we herein present our diversity levels instead by state, which our genetic structuring methods identified as significantly different clusters. Diversity levels were quite similar between states (Table 5.5). Utah, with only two occurrences, still had only marginally lower numbers for total alleles, effective alleles, and Shannon index than Colorado, with its

many occurrences. However, Utah's plants had only 1.2 alleles per locus that were unique to the state as compared to the 8.2 of Colorado, although individual occurrences in both states had similar numbers of private alleles.

Table 5.4: Table of occurrence and statewide summary statistics as calculated by GenAlEx. All of the summary statistics were calculated independently per locus by population and then averaged across loci, which is reported here alongside the standard error of the mean. States were calculated separately, as singular entities, rather than a sum or an average of their constituent occurrences. Na is the allelic richness. Ne is the number of effective alleles, calculated as the reciprocal of the Simpson index. I is the allelic diversity as calculated by the Shannon index. Ho is observed heterozygosity and He is expected heterozygosity, calculated as one minus the Simpson index. F is the inbreeding coefficient, calculated as one minus the ratio of observed to expected heterozygosity. Pa is the number of private alleles, i.e. the number of alleles possessed by that occurrence and no other occurrences.

Population		Na	Ne	I	Ho	He	F	Pa
4210	Mean	6.167	3.500	1.251	0.526	0.603	0.092	0.667
	SE	1.352	0.677	0.289	0.114	0.127	0.128	0.333
8423	Mean	7.833	5.220	1.544	0.493	0.672	0.221	1.167
	SE	2.072	1.422	0.352	0.085	0.116	0.079	0.477
9263	Mean	6.333	3.290	1.306	0.562	0.631	0.143	0.167
	SE	1.229	0.516	0.217	0.099	0.089	0.090	0.167
14908	Mean	5.833	4.283	1.330	0.595	0.618	0.044	0.167
	SE	1.470	1.176	0.333	0.141	0.133	0.077	0.167
6368	Mean	7.333	3.161	1.338	0.516	0.625	0.149	1.500
	SE	1.783	0.490	0.227	0.079	0.076	0.105	1.147
7630	Mean	4.167	2.439	0.925	0.408	0.482	0.067	0.167
	SE	0.601	0.494	0.215	0.094	0.113	0.125	0.167
9127	Mean	4.333	2.274	0.959	0.519	0.504	-0.038	0.500
	SE	0.615	0.287	0.179	0.095	0.094	0.051	0.224
Colorado	Mean	9.500	4.314	1.506	0.484	0.653	0.225	8.167
	SE	2.262	0.954	0.302	0.071	0.112	0.071	2.638
3828	Mean	5.667	3.430	1.249	0.569	0.613	0.036	0.500
	SE	1.022	0.661	0.242	0.088	0.106	0.062	0.224
28883	Mean	4.833	3.298	1.232	0.534	0.640	0.130	0.667
	SE	0.703	0.484	0.180	0.091	0.083	0.118	0.494
Utah	Mean	7.167	3.649	1.410	0.538	0.675	0.190	1.167
	SE	0.792	0.598	0.171	0.073	0.065	0.094	0.477

Morphological characters

The range of variation we measured in calyx teeth was considerable. The average plant had calyx teeth that averaged 1.52mm long but had individual teeth that ranged from 1.16mm-1.88mm. For 20% of the plants, the largest tooth was more than 1mm larger than the smallest tooth, and three plants (one from each geographic region) spanned more than 1.3mm between the smallest and largest tooth. The range of variation we found in tooth size within a single plant alone was greater than most of the published ranges for either species in its entirety (Table 5.2).

The calyx tooth ranges we measured were substantially larger than the more conservative ranges published, and even marginally larger than Welsh's more broad ranges (Table 2). Individuals of *A. raphaelensis* had teeth that ranged from 0.71-2.20mm, while those from *A. linifolius* ranged from 0.71-3.25mm (or 0.85-3.25mm if we exclude Dolores River valley plants that were potentially *A. raphaelensis* plants "misidentified" as *A. linifolius*). Just five individual teeth in *A. linifolius* (from just two plants) exceeded the 2.8mm maximum cited by Barneby (1964) and Welsh (2007). The mean tooth length for *A. raphaelensis*, 1.32mm, was slightly but significantly shorter than that of *A. linifolius* at 1.69mm ($P<0.01$, $F=10.09$, $df=1$) (Figure 6). Similarly, there was a slight but significant difference in calyx tooth length between the clusters identified by our genetic analyses, with plants from Utah having smaller teeth than those from Colorado (1.24 and 1.60, respectively, $P<0.01$, $F=10.09$, $df=1$).

Notably, there was no significant difference between the calyx teeth of plants from the Dolores River valley that had previously been identified as *A. raphaelensis* and those identified as *A. linifolius* ($P=0.96$).

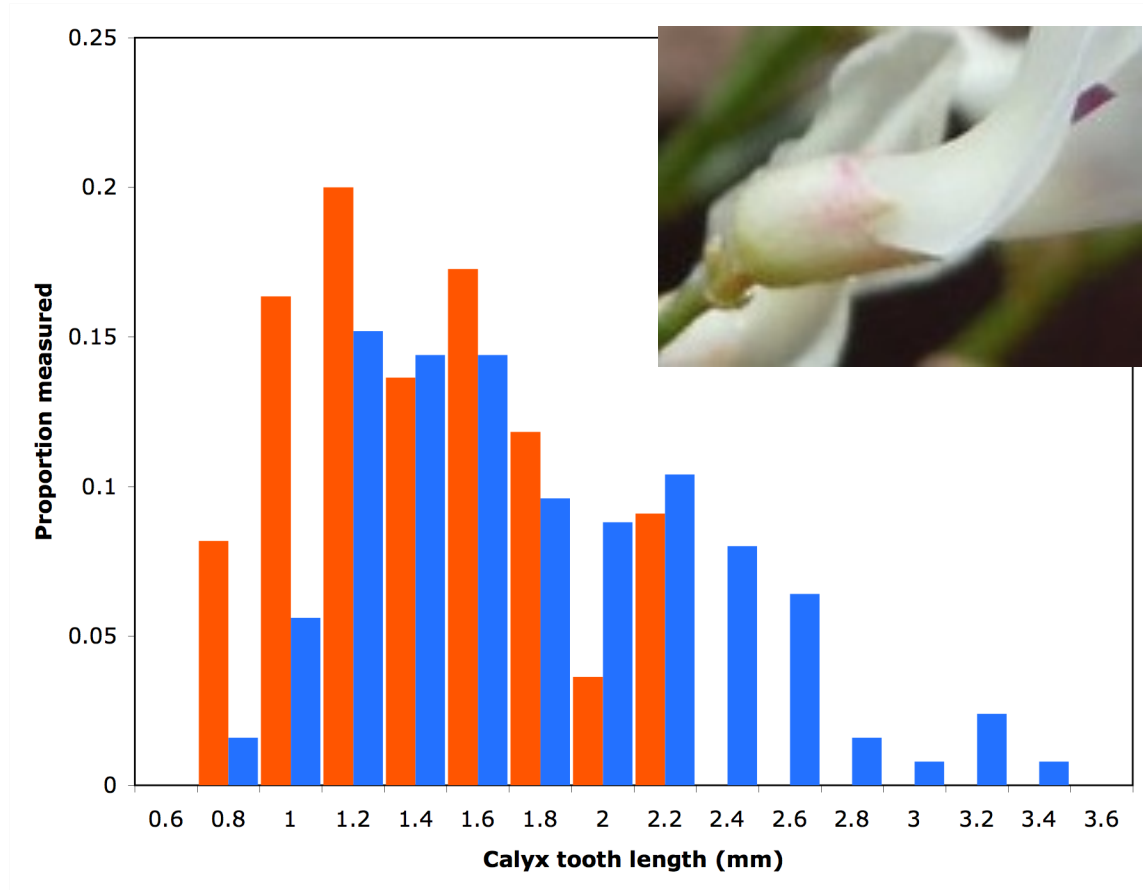


Figure 5.6: Histogram of calyx tooth lengths of *Astragalus linifolius* (blue, $n=125$) and *Astragalus raphaelensis* (orange, $n=110$), with inset of *A. linifolius* calyx, demonstrating the high degree of variation in calyx tooth length within a single individual (Photo courtesy of Lori Brummer, CNHP 1997+). There was a significant difference in calyx tooth length between species ($P<0.01$), with the mean size in *A. linifolius* being larger than in *A. raphaelensis*, largely due to the right skew. However, all *A. raphaelensis* teeth fall within the range of variation of *A. linifolius*.

Discussion

Taxonomy

Genetic considerations

The four independent methods that we used to assess genetic structure within the species (PCoA, cluster analysis, STRUCTURE, and AMOVA) are all in agreement that a) there are two main genetic clusters within the sampled occurrences and b) these two genetic clusters closely correspond to the Colorado and Utah groups of occurrences, not the *a priori* species. Although STRUCTURE assigned the western-most Colorado occurrence to the Utah cluster, PCoA, cluster analysis, and AMOVA all place it in the Colorado cluster. All four analyses agree that the occurrences in Colorado are not statistically different from one another. The occurrences in Utah are somewhat distinct from those in Colorado according to all four analyses, but according to the partial Mantel test, there is no difference between states once geographic distance is accounted for. Thus, because geographic distance alone is statistically sufficient to explain the difference between states, there is no need to invoke additional reproductive isolating barriers to explain the genetic structuring we observed. In other words, the structure we observed matches what would be expected for one disjunct species, rather than two isolated species.

Furthermore, our F_{ST} overall of 0.23, even when including both Utah and Colorado collections, is well within the range found in other species of *Astragalus*. The narrow endemic *Astragalus albens* Greene has a very low overall θ_P ($\sim F_{ST}$) of 0.01 (via allozymes), although the entire species inhabits an area less than 23km across (Neel,

2008). Similarly, the US federally endangered *Astragalus holmgreniorum* Barneby has an only somewhat higher F_{ST} of 0.09 (via microsatellites) across a range less than 25km in diameter (King et al., 2012). By comparison, the two *A. rafaensis* occurrences in the San Rafael Swell alone are more than 26km apart and have a pairwise F_{ST} of 0.10. On the other hand, the ultra-rare Grand Canyon endemic *Astragalus cremnophylax* Barneby var. *cremnophylax* has a staggeringly high overall F_{ST} of 0.44 (via AFLPs) for a species that spans less than 18km (Travis et al., 1996). The critically endangered Spanish species *Astragalus nitidiflorus* Jiménez & Pau has an θ_{ST} of 0.26 (via ISSRs) across an area hardly more than 3km in diameter (Vicente et al. 2011). The somewhat more widespread Idaho endemic *Astragalus oniciformis* Barneby has an F_{ST} of 0.113 (via ISSRs) across its roughly 80 kilometer range (Alexander et al., 2004), while the comparative generalist *Astragalus filipes* Torr. ex A. Gray has a species-wide AFLP F_{ST} of 0.27 across populations up to 1350 km apart (Bushman *et al.*, 2010). The total overall F_{ST} of 0.23 we observed is thus unremarkable within the genus, especially given the relatively broad span of roughly 225 kilometers between the most distant occurrences.

Although the Utah occurrences are moderately and significantly different from those in Colorado, those differences are fully accounted for by geographic distance, which alone explains more than 75% of the variance in genetic difference between all occurrences. Therefore, we recommend that *A. linifolius* be subsumed into *A. rafaensis*. Henceforth in this document, we will refer to the historical, conventional species as *A. rafaensis* s.s. (*sensu stricto*) (for all plants west of the Uncompahgre Plateau) and *A.*

linifolius (for all plants east of the Uncompahgre Plateau). We will refer to the combination of both *A. raphaelensis* s.s. and *A. linifolius* as *A. raphaelensis* s.l. (*sensu lato*).

Morphological characters

Based on the type specimen descriptions, only one morphological characteristic separated *A. linifolius* from *A. raphaelensis* s.s.: flower color (Jones, 1923; Osterhout, 1928). Both pod orientation and calyx tooth length were more recently proposed, although both are somewhat contested among leading authorities (Table 5.2). The primary character, flower color, is problematic in both its subjectiveness and its patchy distribution. Although pink-purple may seem easily discernable from white, many *A. raphaelensis* s.s. plants have flowers that are “barely colored”, and so pale pink as to be nearly white, even in the heart of the San Rafael Swell in Utah (Jones, 1923) (Figure 5.1). Particularly along the Dolores River in Colorado, at least several occurrences have both individuals with pure white flowers and individuals with pink-purple flowers growing together in the same occurrence (Table 5.1) (SEINet, CNHP, 2013).

The second trait, pod orientation, also appears to be problematic. Most authorities generally claim that *A. linifolius* has strictly upright pods while *A. raphaelensis* has strictly reflexed pods (Table 5.2). However, many plants, particularly (but not exclusively) along the Dolores River, have both erect and reflexed pods on the same plant. In fact, even a few plants on the east side of the plateau, in what has generally been thought of as strict *A. linifolius* habitat, have some reflexed pods (Statwick, personal observation, Figure

5.7). Still, perhaps the most damning evidence against this characteristic is actually Osterhout's initial field description of the pods of *A. linifolius* as “not upright” (SEINet).



Figure 5.7: Close-up of pods from *Astragalus linifolius* observed at site 14908, just ~15km south of the type location in Grand Junction. *A. linifolius* is generally considered to have strictly erect pods, but this particular individual and others in the area have both erect and reflexed pods (indicated by *). Similarly, many plants in the Dolores River valley have both erect and reflexed pods on the same plant (inset).

Finally, the third trait, calyx tooth length, does have a significantly different mean between *A. rafaensis* s.s. and *A. linifolius*. However, we feel that this is a classic case of when statistical significance does not correspond to biological significance. Because the range of tooth sizes for *A. rafaensis* s.s. falls almost entirely within the range of variation for *A. linifolius* (even excluding potentially “misidentified” specimens), this trait is not a useful keying characteristic. Of the specimens we measured, more than 83%

of individual calyx teeth and 56% of plants - including both holotypes - fall into the overlap zone and thus be morphologically ambiguous by this trait alone. Oddly enough, the *A. linifolius* holotype actually has calyx teeth that, at 1.26mm, are slightly smaller than those of the *A. rafaensis* holotype, at 1.40 mm. Also, this trait, even if it were a good character, is in conflict with flower color, as the herbarium specimens collected along the Dolores River valley which have been identified as *A. linifolius* on the basis of color have calyx teeth that are no different from purple flowered individuals from the same region.

In short, pure white flowers exist west of the Uncompahgre Plateau, pods can be both erect or reflexed (or both) on either side of the plateau, and calyx teeth are marginally longer in the eastern populations, but with substantial overlap in sizes. Thus, there appears to be no single morphological trait or combination of morphological traits that discretely delineates between the *a priori* species. Also, no traits discriminate between the genetically identified clusters of Colorado and Utah. Thus, we believe that the white flowers, erect pods, and slightly longer calyx teeth characteristic of what has been called *A. linifolius* represent nothing more than continuous regional variation within *A. rafaensis s.l.* The species can therefore have white to light pink-purple flowers, erect or pendulant pods, and calyx teeth from less than 1mm to more than 3mm.

Given this range of morphological variation within *A. rafaensis s.l.*, we recommend that *A. toanus* and *A. saurinus* be examined in the future, to determine whether they should still be considered distinct species or reduced to varieties or subspecies.

Conservation

Inbreeding and genetic diversity

Plants are generally considered moderately inbred when their inbreeding coefficients exceed 0.25 (Ritland 1996). Only three occurrences had F_{IS} values significantly greater than zero, even then, the largest inbreeding coefficient was only 0.22. Since the three occurrences with non-zero inbreeding coefficients also happen to be among the most diverse, it is possible that these high F_{IS} values are not a symptom of inbreeding, but rather an indication of a lack of Hardy-Weinberg equilibrium due to gene flow and/or mutation. New alleles introduced to the occurrence via one of these mechanisms would quickly increase the genetic diversity of the occurrence, but also the F_{IS} , at least for the first few generations when a new allele would be limited to a novel plant and its offspring.

Thus, inbreeding seems not to be an immediate concern for any of the occurrences we sampled, and populations are likely genetically stable in the short to medium term. Because we would not necessarily predict such high diversity and low inbreeding given the known range and life history of the species, it is possible that the species is much more abundant than it appears.

Reliability of known occurrences

While we collected tissue from less than a third of the 49 total occurrences of *A. rafaensis* s.l. according to NatureServe (2015), it is likely that this reported number is

substantially inflated. For *A. linifolius*, several occurrences are suspect. The description for the type location – “Hills approximately 6 miles across the Colorado River from Grand Junction” - is vexingly vague, and is likely to be a duplicate of one of the other, more recently documented occurrences in the area, such as site 8267. A second occurrence, in Dominguez Canyon, was both discovered and last seen in 2010. It had just nine individuals at the time and was in a very atypical habitat (a damp, wooded floodplain rather than a dry and sparsely vegetated slope) (CNHP, 2013). Since we did not find any evidence of an occurrence at that location, it is possible that this was an ephemeral occurrence, temporarily established by one of the larger, well known occurrences further up the canyon. Also, four of the historical occurrences of *A. linifolius* are located along the Dolores river, and have generally been considered to be “misidentified” *A. raphaelensis* s.s. (Figure 5.2). Only one of the four is not already sympatric with other tracked *A. raphaelensis* s.s. occurrences, so unfortunately this does little to bolster the known range *A. raphaelensis* s.l. Thus, all told, there are likely just 15 or possibly 16 occurrences of what has been called *A. linifolius*, rather than 21.

Occurrence numbers of *A. raphaelensis* s.s. are likely overestimated as well. In particular, the 21 known occurrences in Utah are based on point observation records, rather than mapped occurrences (as in Colorado), and most of the records seem to be identifying the same two occurrences. Similarly, many of the collection location descriptions of herbarium specimens don’t match the georeferenced locations, such that four specimens from the same collector with nearly identical verbatim descriptions are georeferenced to four different locations as much as 100km apart (Seinet, 2015). After

careful consideration and some field verification of the records, we think that there are no more than three (or perhaps four, if one were to split the two sub-occurrences of 3828 into separate occurrences) known occurrences in Utah, including one along the Dolores River, barely across the border from Colorado. Combined with the eight known occurrences in Colorado (including those “misidentified” as *A. linifolius*), there are only 11 reliably documented occurrences of *A. rafaensis* s.s., rather than 28.

Since we are recommending that *A. linifolius* be subsumed into *A. rafaensis* s.l., the known number of occurrences increases to approximately 26 (from 11 for *A. rafaensis* s.s.), but is substantially less than the 49 recognized by NatureServe (2015), and even less than the 28 that were previously thought to exist for *A. rafaensis* s.s. alone. We are optimistic that there are other undocumented occurrences, at least along the Uncompahgre Plateau and within the San Rafael Swell, which may bolster the size, if not the range, of the species. Based on geology and topography, there is likely to be substantial suitable habitat for this species that remains largely inaccessible and thus unexplored. Also, several non-georeferenced herbarium records have vague yet tantalizing descriptions that seem to indicate the possibility of one or more additional occurrences within the San Rafael Swell. Still, we did explore a few known seleniferous areas in the southern half of the San Rafael Swell and along the Poison Strip, halfway between the San Rafael Swell and Dolores River valley occurrences, but found no *Astragalus rafaensis*, despite finding other seleniferous *Astragalus* and *Stanleya* species.

Threats to known occurrences of *A. rafaensis* s.l.

We observed several potential threats to the current and future viability of *A. rafaensis* occurrences when collecting tissue from these occurrences.

The occurrences within Colorado are mostly relatively remote and inconspicuous. Many of the occurrences are only accessible via current and former access roads for ranching and/or uranium and vanadium mining, and then often only with a high-clearance 4WD vehicle. Furthermore, the occurrences are often a distance off the road on a footpath. Still, continued ranching, mining, and natural gas extraction will likely pose an ongoing threat to at least some occurrences. In addition, outdoor recreation such as camping, hiking, and OHV use may pose a threat to certain Colorado occurrences, particularly those in the Cactus Park zone of the Dominguez-Escalante National Conservation Area. We observed a number of plants in close proximity to dirt roads, trails, and dispersed campsites in this area.

A few occurrences, particularly along the Dolores River and immediately south of Grand Junction, are adjacent to paved roads. However, these occurrences are generally on steep washes or embankments, and are not close to towns or other landmarks that would encourage spontaneous visitors. Although inadvertent foot traffic through these areas is probably minimal, illegal dumping and/or littering appears to be at least a minor issue at a few sites.

In the San Rafael Swell, occurrences are readily accessible to the public. The smaller occurrence, 28883, is likely less threatened. Although it occurs in a gully that runs parallel and immediately adjacent to a road, that road is rougher and less frequently

traveled than many of the others within the Swell, and the occurrence is not near any tourist landmarks. The larger occurrence, which spans several proximate canyons, including Calf Canyon, Buckhorn Wash, and the Little Grand Canyon, has considerably more evidence of threat. It is near the San Rafael Campground, the Paleolithic rock art in Buckhorn Wash, and other geologic formations that attract visitors. The individuals in Calf Canyon are near OHV trails and dispersed campsites. Those in Buckhorn Wash are bordering one of the most well-traveled roads in the Swell (Figure 5.8). The plants in the Little Grand Canyon are immediately adjacent to a footpath with evidence of heavy hiker and cattle traffic. They are also within meters of tamarisk thickets (*Tamarix sp.*) and the soil near the tamarisk is visibly crusted with evaporite salts, although the *A. raphaelensis* are typically upslope from the salt accumulation. On the other hand, the hydrological conditions that create evaporite salts may simultaneously enrich soil bioavailable selenium concentrations, perhaps creating a more favorable habitat for *A. raphaelensis* (Statwick, Unpublished Data), at least in the short term.

Despite evidence of cattle ranching at or near several occurrences throughout the range of *A. raphaelensis*, grazing is unlikely to be a significant threat. Because of its seleniferous habit, the plant is malodorous, unpalatable, and extremely toxic (Trelease and Trelease, 1937). Trampling is likely to be a bigger threat than grazing, although the plants can be more than half a meter tall and are rather tough and fibrous. Given the choice, hikers, grazers, and OHVs are more likely to go around the plants than over them, but some incidental trampling is probable, particularly in high traffic areas.



Figure 5.8: A mature *Astragalus rafaensis* individual (indicated by arrow) growing in very close proximity to a well-traveled road in the San Rafael Swell, Utah. Despite its exposed location, this plant is likely quite old, given the very substantial thatch at the base of the plant from previous years' growth (indicated by *).

Invasive plants, particularly *Bromus tectorum* or cheatgrass, are present at many of the sites, even some of those that appear relatively undisturbed otherwise. Given the known ecosystem engineering capabilities of some invasive species, particularly the intensification of fire by cheatgrass and the salinification of soil by tamarisk, we suspect that the proliferation of invasive species, especially as facilitated by humans and livestock, is a primary threat to the species. Fortunately, the seleniferous habitat of *A. rafaensis* makes it less likely that invasive plants will be able to outcompete it on its native seleniferous soils. The soils are already stressful or toxic to generalist plants, but

Astragalus hyperaccumulators seem able to further toxify the soil by exuding highly bioavailable forms of organic selenium from their roots, possibly acting as a form of competitive inhibition or elemental allelopathy (El Mehdawi *et al.*, 2012). Nonetheless, altered landscape-scale ecosystem processes may still negatively affect the species.

Climate change is considered a serious threat to all of the species and habitats within Colorado (CPW, 2015). As a drought adapted species, *A. rafaensis* may be less vulnerable to changing precipitation regimes. However, since *A. rafaensis*, like other hyperaccumulators, seems to be endemic to seleniferous soils, it may not have the ability to move up in elevation or latitude as the climate warms. Still, there is evidence that other edaphic endemics have endured substantial climate change with little or no change in range, perhaps because such endemics are much more sensitive to soil than they are to climate (Douglas *et al.*, 2011).

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APPENDIX A: SUPPLEMENTARY FIGURES AND TABLES

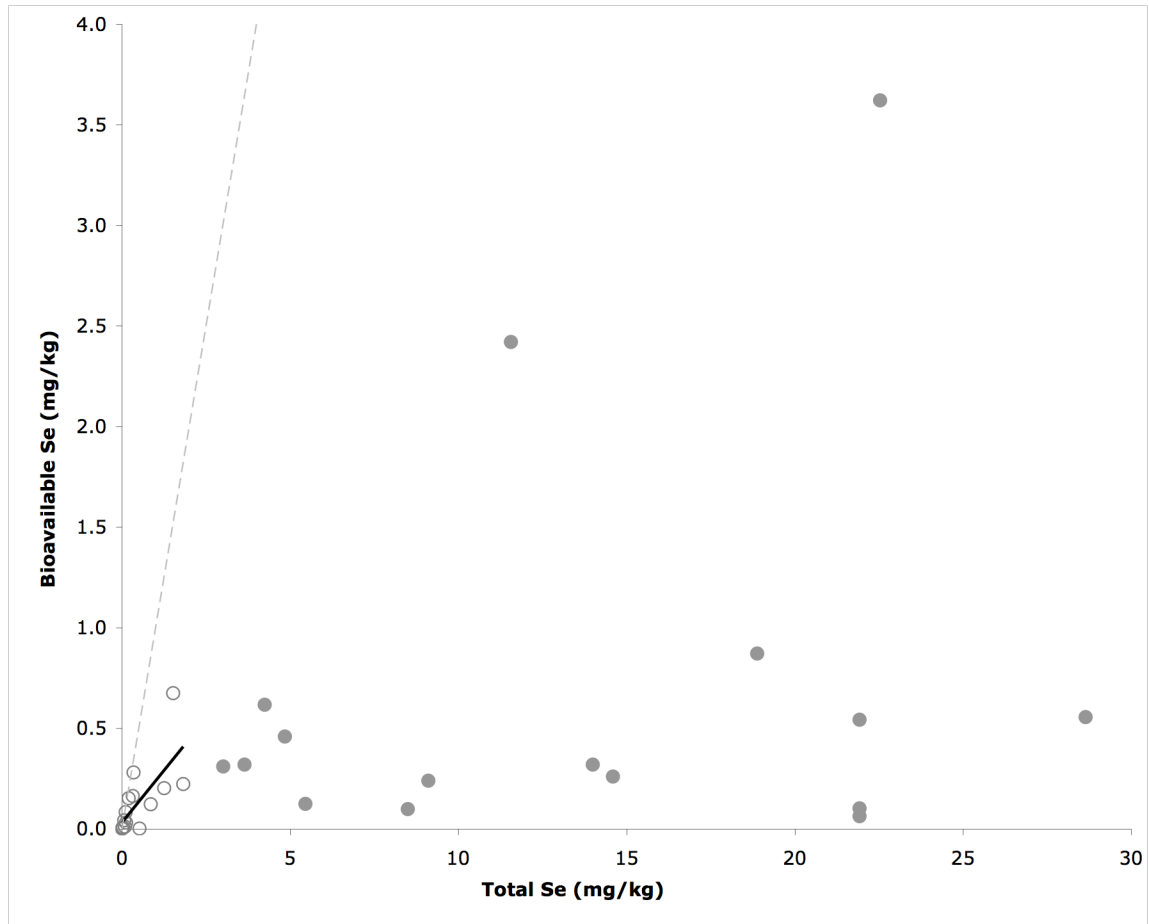


Figure S2.1: Untransformed total soil selenium versus untransformed bioavailable soil selenium. Unfilled circles represent normal soils, i.e. those with $<2\text{mg/kg}$ total Se. The solid line represents the best fit line for normal soils. The filled circles represent seleniferous soils ($>2\text{mg/kg}$ total Se). There is no trendline for seleniferous soils because the relationship is not significant. The dashed light grey line represents the identity line, i.e. a 1/1 bioavailable fraction.

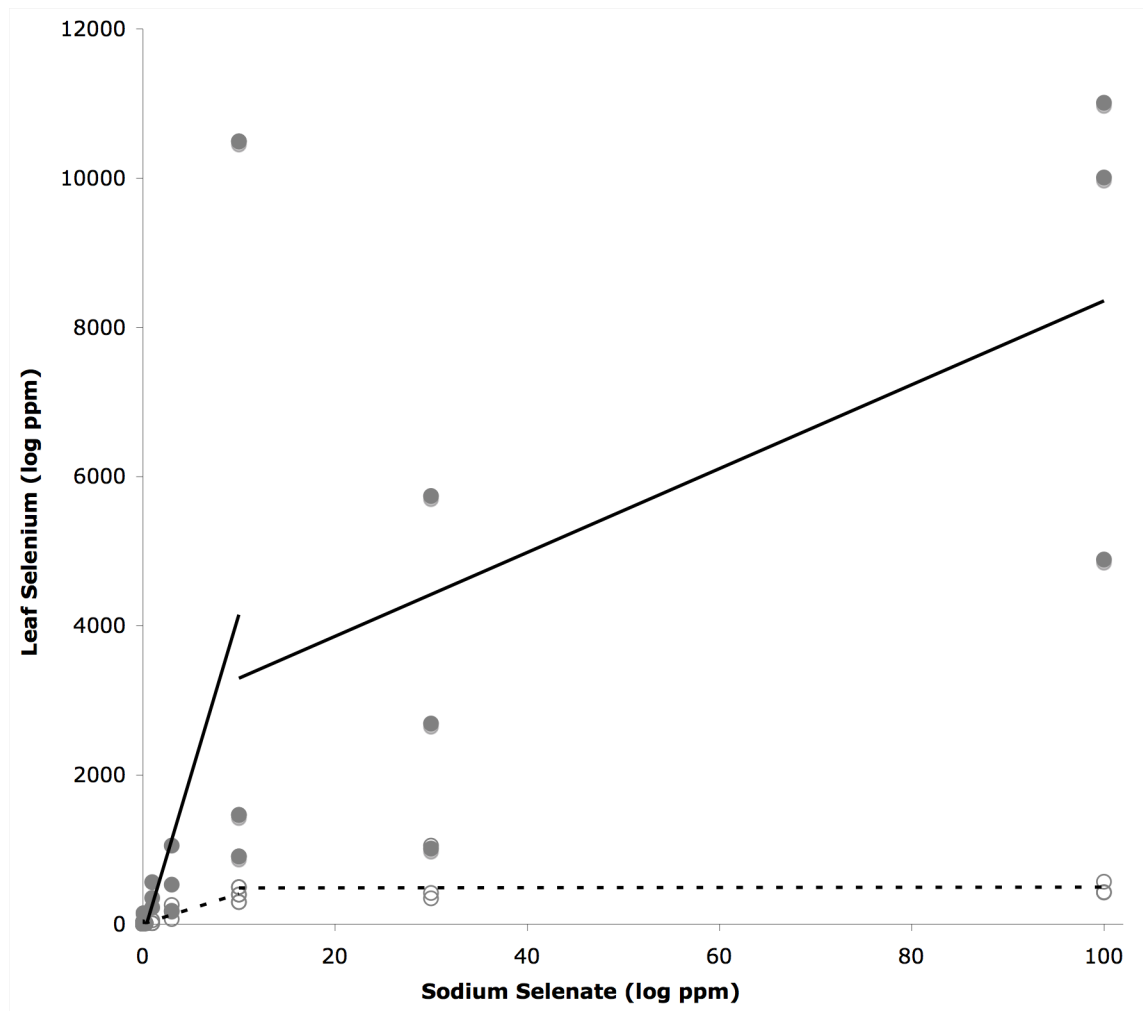


Figure S3.1: Accumulation curves of untransformed whole leaf selenium concentration versus untransformed soil dosage of sodium selenate. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Linear regression analyses were broken into three segments, 0-0.1mg/l, 0.1-10mg/l, and 10-100mg/l. Solid lines represent best fit lines for *A. bisulcatus* and dashed lines represent best fit lines for *A. cicer*.

Table S3.1: ANCOVA table of species and log-transformed tissue concentration on plant performance metrics. Non-significant p-values are grayed.

	Species	Tissue Se	Species* Tissue Se	n	R ² adj.
Aboveground Mass	P<0.001	P<0.001	P=0.17	72	0.53
Belowground Mass	P<0.001	P=0.60	P<0.05	72	0.48
Root/Shoot Ratio	P<0.01	P<0.01	P<0.01	72	0.28
Net Leaf Proportion	P<0.05	P=0.06	P<0.05	72	0.20
Net Stem Growth	P=0.16	P=0.21	P=0.10	72	0.07

Table S5.1: The loci and modifications to PCR conditions used in this study. The primers and standard PCR conditions are described in King et al. (2012). Because we used universal primer complexed dyes to visualize our fragments, we added universal primer tags to the 5' end of either the forward (F) or reverse (R) primer for each locus.

Locus	Anneal Temp °C	MgCl μ l	BSA μ l	Tag
AhoA1	68	0.8	0	CAGT – F
AhoA2	61	0.8	0	T7 – F
AhoD108	53	0.5	0.06	M13R – R
AhoD128	53	0.8	0	M13R – R
AhoD136	61	1	0.06	CAGT – R
AhoD147	61	1.5	0.06	T7 – F

Table S5.2: Pairwise matrices of F_{ST} (top) and D (bottom) values across all sampled occurrences with >20 individuals. The first four occurrences listed (4210-14908) are regarded as *A. linifolius* and were collected in the Gunnison River valley; the next three (6368-9127) are generally regarded as *A. raphaelensis* and were collected in the Dolores River valley; and the final two (3828 and 28883) are regarded as *A. raphaelensis* and were collected in the San Rafael Swell.

F_{ST}	4210	8423	9263	14908	6368	7630	9127	3828	28883
4210	0.000								
8423	0.062	0.000							
9263	0.059	0.059	0.000						
14908	0.068	0.040	0.056	0.000					
6368	0.109	0.075	0.080	0.082	0.000				
7630	0.119	0.087	0.112	0.083	0.123	0.000			
9127	0.119	0.076	0.089	0.076	0.100	0.133	0.000		
3828	0.232	0.199	0.172	0.209	0.142	0.253	0.239	0.000	
28883	0.181	0.170	0.172	0.158	0.144	0.219	0.205	0.101	0.000

D	4210	8423	9263	14908	6368	7630	9127	3828	28883
4210	0.000								
8423	0.296	0.000							
9263	0.234	0.327	0.000						
14908	0.267	0.163	0.225	0.000					
6368	0.458	0.366	0.390	0.316	0.000				
7630	0.461	0.307	0.428	0.294	0.479	0.000			
9127	0.550	0.333	0.352	0.307	0.364	0.484	0.000		
3828	1.224	1.146	0.763	0.973	0.557	1.080	1.062	0.000	
28883	1.078	1.230	1.170	0.838	0.724	1.041	1.073	0.469	0.000

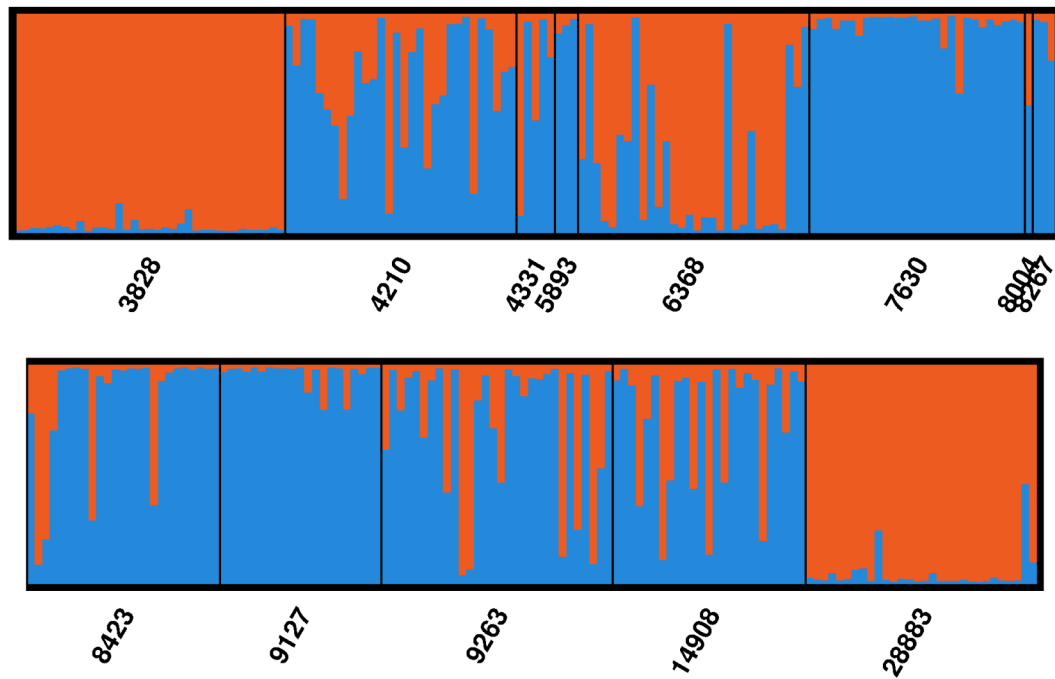


Figure S5.1: STRUCTION diagram of major group output from CLUMPAK. The blue cluster largely predominates the genotype of individuals in Colorado and the orange cluster predominates the genotype of Utah individuals, although many Colorado individuals and collection sites contain substantial Orange signal, particularly 6368, which has a majority orange signal.